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Department of Microbiology and Immunology
Uniformed Services University of the Health Sciences**

Abstract

Title of Dissertation:

Mutations in the Histone-like Nucleoid Structuring Regulatory Gene (*hns*) Decrease the Adherence of Shiga Toxin-producing *Escherichia coli* 091:H21 Strain B2F1 to Human Colonic Epithelial Cells and Increase the Production of Hemolysin

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Candidate, Doctor of Philosophy, 1999

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Non-0157 serotype Shiga toxin-producing *E. coli* (STEC) cause a significant amount of food-borne hemorrhagic colitis (HC) and life-threatening hemolytic uremic syndrome (HUS) worldwide and yet these strains do not encode intimin, a major adherence factor encoded by the most common serotype 0157:H7 isolated from documented cases of HC and HUS. In this investigation, we attempted to isolate specific adhesins critical for attachment of intimin-negative STEC 091:H21 to human colonic epithelial cells. Transposon mutagenesis of B2F1 was accomplished with the mini-Tn5*phoACm'* mobile element and a mutant bank of B2F1 colonies that carried putative in-frame PhoA-positive transposon insertions was isolated. The bank was screened for mutants unable to adhere to the human colonic epithelial

T84 cell line in both its nonpolarized and polarized states. An adherence mutant designated as 34.7 was identified that did not bind to these cells. Subsequently, mutant 34.7 was found to contain an out-of-frame insertion of the transposon into *hns*. The *hns* gene encodes the H-NS protein that has been reported to modulate the expression of various housekeeping as well as virulence-associated genes encoded by *E. coli* and other gram-negative organisms. Disruption of *hns* in 34.7 not only caused reduced adherence to T84 cells but resulted in overproduction of STEC-hemolysin encoded on the large ~90 kb plasmid carried by this strain. When H-NS was supplied to 34.7 *in trans* on a moderate copy number plasmid, both the reduced adherence phenotype and the hyper-hemolytic phenotype reverted to wild-type. Because hemolysin can destroy the T84 monolayer over the course of the adherence assay, it was difficult to assess the direct effect of the *hns* mutation on adherence of B2F1 to T84 cells. Hence, we sought to separate the reduced adherence phenotype from the hyper-hemolytic phenotype exhibited by mutant 34.7. To accomplish this, an attempt was made to inactivate *hns* in the plasmid cured derivative of B2F1, strain S11. The plasmid-cured strain attached to T84 cells with a wild-type adherence phenotype. The *hns* gene encoded by S11 was partially inactivated by disruption of *hns* by insertion of a suicide plasmid. The resultant *hns* mutant, S11366.15, was unable to bind to T84 cells and the reduced adherence phenotype was complemented *in trans* with wild-type H-NS expressed from a low copy number plasmid. Hence, mutations in *hns* that ablated or reduced the activity of H-NS directly affected the capacity of B2F1 or its plasmid-cured derivative, S11, to adhere to T84 cells. These data suggests that, in B2F1, H-NS may coordinately regulate the expression of adherence factors and the production of hemolysin.

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Escherichia coli 091:H21 Strain B2F1 to Human
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the Production of Hemolysin**

by

Maria Elizabeth Scott

**Dissertation submitted to the Faculty of the
Department of Microbiology and Immunology Graduate Program
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**Dedicated
To Dr. Henry Wu**

**Your intelligence was wrapped in warmth
and the umbrella of your kindness will be felt forever.
I will miss you and never forget you.**

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INTRODUCTION

I. Preface

For the purpose of this dissertation, *Escherichia coli* that produce one or more types of Shiga toxin (Stx) will be called STEC whether or not they produce the adhesin intimin. Intimin is the primary intestinal cell adhesin produced by *Escherichia coli* 0157:H7 strains that are responsible for most cases of hemorrhagic colitis and hemolytic uremic syndrome in developed countries. The subset of STEC that carry a pathogenicity island called the locus of enterocyte effacement (LEE) and express intimin encoded in LEE will be referred to as enterohemorrhagic *Escherichia coli* (EHEC).

II. Purpose and overview

Shiga toxin-producing *E. coli* (STEC) serotype 091:H21 strain B2F1 was originally isolated from the stools of a 3 year old with kidney failure associated with the hemolytic uremic syndrome (Dr. M. Karmali, personal communication). This human pathogen does not encode intimin. The genes that encode colonization adherence factors for strain B2F1 and for other intimin-negative STEC have not been described. The purpose of this thesis was to define factors important for adherence of this strain and other intimin-negative non-0157 strains to human epithelial cells. During this investigation we found that H-NS, a histone-like protein, plays a critical role in the adherence of B2F1 to the human colonic epithelial cell line T84.

In the next sections, I will provide background information on several topics that are integral to my study. First, I will present a summary of the characteristics of the various classes of diarrheagenic *E. coli*. I will emphasize enteropathogenic *E. coli* (EPEC) and STEC, and I will provide a detailed description of the shared and unique features of these two groups. Second, I will describe important attributes of the histone-like nucleoid-structuring protein, H-NS, that regulates the expression of various genes encoded by several strains of *E. coli*, and other gram-negative bacteria.

III. Overview of enteric pathogenic *Escherichia coli* implicated in diarrheal disease.

E. coli were initially thought to be benign commensals of the human intestinal tract, but in the 1940s researchers discovered that some isolates of *E. coli* that infect the bowel are in fact frank pathogens (Bray, 1945). Currently, 6 categories of *E. coli* that cause distinct diarrheal disease have been identified (Levine, 1987; Saravino et al., 1993). These groupings are based on: 1.) features of disease manifested; 2.) specific virulence determinants associated with the infecting strain; 3.) nature of the interactions of the organism with the intestinal mucosa, and; 4.) serotype of the strain (Levine 1987). The six classes of diarrheagenic *E. coli* include: 1) enterotoxigenic (ETEC); 2) enteroinvasive (EIEC); 3) enteroadherent-enteroaggregative (EaggEC); 4) enteroadherent-diffusely adherent (DAEC); 5) enteropathogenic (EPEC); and, 6) enterohemorrhagic (EHEC), a subset of (STEC).

A. Enterotoxigenic *E. coli* (ETEC).

ETEC are one of the most common causes of diarrhea among travelers in developing countries (Levine. 1987). ETEC are also responsible for significant morbidity and mortality, especially among infants and elderly persons in developing countries. ETEC produce heat-labile enterotoxin (LT), heat- stable enterotoxin (ST), or both (Weikel et al., 1986). The heat-labile toxin produced by ETEC organisms is homologous to the toxin encoded by *V. cholerae*. These toxins are the primary cause of the watery diarrhea associated with ETEC infections. ETEC elaborate several colonization factors, but the adhesins known to be important in human infections include CFA/1, CFA/11, E8775, PCF0159, and AAF/1, and perhaps the longus pilus encoded by *lngA* (Giron et al. 1994).

B. Enteroinvasive *E. coli* (EIEC).

EIEC resemble *Shigella* in that they invade and multiply within epithelial cells and cause severe abdominal pain, watery diarrhea, and sometimes dysentery. The high number of leukocytes in the stools of infected individuals is a hallmark of EIEC infection (Dupont et. al., 1971). EIEC carry a large plasmid that encodes genes necessary for invasion, and this plasmid is similar to the virulence-associated plasmid of *Shigella* (Hale et al., 1985). Enterotoxic and cytotoxic activities are present in culture filtrates and cell lysates of EIEC strains (Fasano et al. in 1990). Shiga toxin type 1 (Stx1) is not responsible for this activity because antiserum specific for Stx1 does not neutralize the EIEC toxin activity. The role of the EIEC toxins in pathogenesis is not known.

C. Enteroadherent *E. coli*: enteroaggregative bacteria (EaggEC).

Diarrhea of long duration, greater than or equal to 14 days, in children in developing countries has been linked to the enteroaggregative group of *E. coli* (Savarino, 1993). Enteroaggregative *E. coli* adhere to HEp-2 cells in a stacked brick-like lattice pattern. This pattern of adherence to cells is referred to as aggregative adherence (AA). The aggregative adherence fimbriae is encoded by *aaf/1* (Nataro et al., 1992; Savarino et al., 1994). A heat-stable enterotoxin that is encoded on a plasmid is produced by EaggEC isolates (Savarino et al., 1991; Savarino et al., 1983).

D. Enteroadherent *E. coli*: diffusely adherent bacteria (DAEC).

Epidemiological studies have implicated diffusely adherent *E. coli* as agents of diarrhea in underdeveloped countries (Mathewson et al., 1987). However, factors involved in virulence in humans have not been definitively determined. Two adhesins, F1845 (Bilge et al., 1989) and AIDA-1 (Benz and Schmidt, 1989), are present on some DAEC strains, and the genes from these adhesins have been cloned.

E. Enteropathogenic *E. coli* (EPEC).

1. Characteristics of EPEC

EPEC were isolated in 1945 (Bray, 1945) from an outbreak of infantile diarrhea. In many parts of the developing world, EPEC remains a significant pathogen of children under one year of age. EPEC are also recognized as the cause of protracted and sometimes lethal diarrhea (Robins-Browne, 1987). EPEC do not make LT or ST (Gross et al., 1976). EPEC form microcolonies on the surface of infected epithelial cells by localized

adherence (LA). Initial localized adherence is followed by intimate contact between EPEC and cells that leads to localized degeneration of the epithelial brush border microvilli. This adherence pattern is called an attaching and effacing (A/E) lesion, and it was first described in EPEC-infected gut tissue of piglets (Staley et al. 1969). These observations were subsequently confirmed (Moon et al. 1983; Tzipori et al., 1985) in rabbit and gnotobiotic piglet animal models and in humans infected with EPEC (Ulshen and Rolio, 1980; Rothbaum et al., 1982). HEp-2 cells have been used extensively to detect the localized adherence pattern associated with EPEC (Cravioto et al., 1979). The EPEC- induced A/E lesion (or pedestal) is associated with the assembly of highly organized cytoskeletal structures in the epithelial cells immediately beneath the site of the intimately adherent bacteria (Knutton et al., 1989). Formation of A/E lesions following intimate bacterial attachment to epithelial cells is visualized by staining the polymerized filamentous actin with fluorescein isothiocyanate (FITC)-conjugated to phalloidin, a fungal toxin that binds to polymerized actin (Knutton et al., 1989). This fluorescence actin staining (FAS) test is routinely used to detect EPEC (or EHEC-see below)-mediated A/E activity.

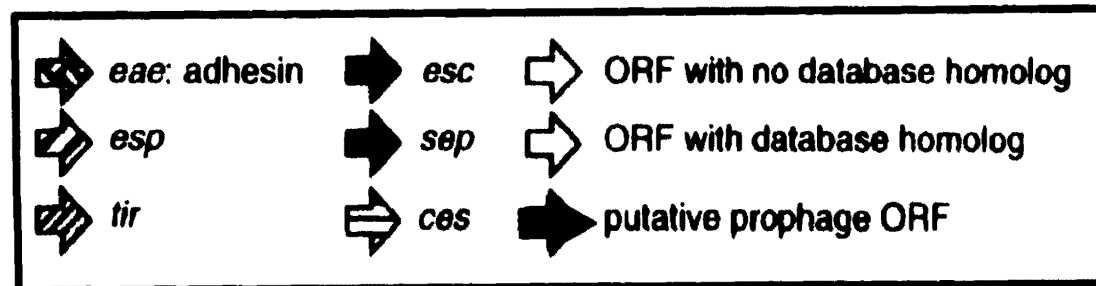
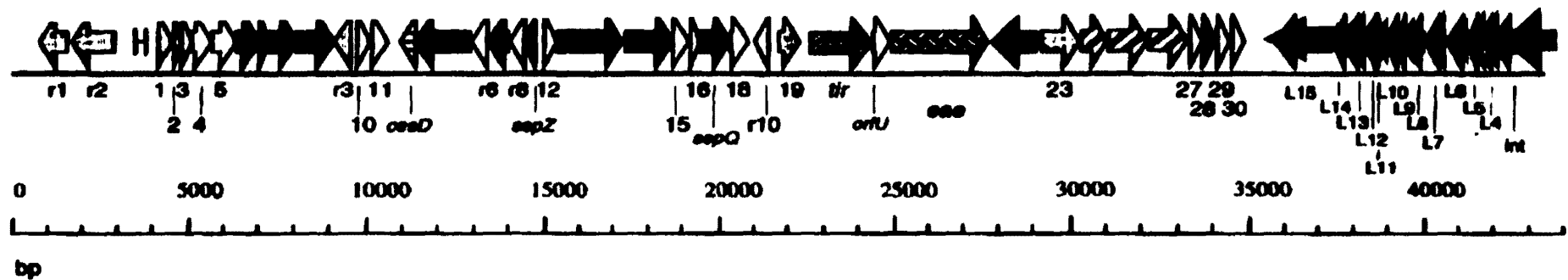
2. *Relationship between A/E lesion formation and the LEE pathogenicity island.*

Formation of an A/E lesion by EPEC is dependent on the product of *eae*, the *E. coli* attaching and effacing gene that encodes a 94 kDa adhesin called intimin (Jerse et al., 1990). In addition, a large plasmid called EAF (EPEC adherence factor) is common to EPEC strains. Pili responsible for the first step in adherence of EPEC to epithelial cells are encoded on the EAF plasmid (Baldini et al., 1983). These pili appear to wrap around each other and

draw bacteria close together; thus, they are called bundle forming pili (*bfpA*) (Giron et al., 1991; Donnenberg et al., 1992; Giron et al., 1993). The plasmid encoded regulatory locus, *per*, is located on the EAF plasmid and controls expression of both the *eae* (for *E. coli* attaching and effacing) and the *bfp* genes. The *eae* gene, whose product intimin promotes close contact of EPEC with host cells (Jerse et al. 1990), resides within a 35 kb region on the chromosome, designated as the locus of enterocyte effacement or LEE (Figure 1). LEE, which is categorized as a pathogenicity island, encodes all the known determinants of the EPEC A/E phenotype (McDaniel et al., 1995; McDaniel et al., 1997). The LEE locus is not present in normal flora *E. coli*, K-12 *E. coli*, or enterotoxigenic *E. coli*, but is found in all EPEC clinical isolates and in the subset of STEC called EHEC (discussed below). The G + C content of the EPEC LEE pathogenicity island is 38.3%, (Elliott et al., 1998), and the rest of the *E. coli* genome is 50.8% G + C (Blattner et al., 1997). This difference suggests that horizontal transfer of the LEE pathogenicity island into *E. coli* from another species of bacteria occurred. The pathogenicity islands of EPEC strain E2348/69 and of EHEC serotype O157:H7 strain EDL933 are inserted into the *E. coli* chromosome at minute 82 of the *E. coli* K-12 chromosome and are downstream of the *selC* locus that encodes the tRNA for selenocysteine (McDaniel and Kaper, 1997). The insertion of the LEE locus in other isolates of EPEC and EHEC can occur at different sites (*pheU*) [Wieler et al., 1997]. Indeed Whittam et al., (1993) reported that the insertion site of the LEE locus correlates with the clonal relatedness of EHEC.

The LEE locus of both EPEC and EHEC is divided into three regions that have known functions. The middle region contains the *eae* gene and the *tir* (for translocated intimin

Figure 1. Map of the locus of enterocyte effacement (LEE). Thin arrows denote the predicted operons. The prophage that is present in EHEC 0157:H7 strains but absent in EPEC E2348/69 is indicated as a solid bar. This diagram is modified from the data of Kaper et al., 1998. Attaching-and-effacing intestinal histopathology and the locus of enterocyte effacement, p., 163-182. In J. B. Kaper, A. D. O'Brien (ed.), *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E. coli* strains.



receptor) gene. Evidence suggest that the *tir* gene encodes a receptor for intimin that is translocated into the host cell during infection (Rosenshine et al., 1996; Kenny et al., 1997). Downstream of *eae* are the *esp* (for *E. coli* secreted proteins) genes that encode secreted proteins responsible for inducing the epithelial cell signal transduction events leading to attachment and effacement of the microvilli. Upstream of *eae* and *tir* are a series of genes [collectively called *esc* and *sep* (for secretion of EPEC proteins)] that encode the type III secretion system important in the extracellular export of the proteins encoded by the *esp* genes (Elliott et al., 1999). The components of the type III secretion system are transcribed from three polycistronic operons designated LEE1, LEE2 and LEE3. The secreted Esp molecules are part of a fourth polycistronic operon called LEE4 (Mellies et al., 1999). The *ler*, for LEE-encoded regulator, activates the expression of LEE2, LEE3 and LEE4, and the expression of *ler* is directly activated by the plasmid encoded *per* gene product. Thus, the expression of the components of the type III secretion system and secreted molecules are controlled by the *per* and *ler* regulatory cascade in EPEC (Mellies et al., 1999). The type III secretions system found in EPEC and EHEC organisms are similar to other specialized protein secretion systems found in a variety of gram-negative human, animal and plant pathogens (e.g. *Shigella*, *Yersinia* and *Erwinia*). The *E. coli* secreted proteins or Esps are made and secreted *in vitro*, but bacterial extracts of these proteins have no effect on the eucaryotic target cell (Jarvis et al., 1995; Jarvis and Kaper, 1996). Only when the Esps are presented to the epithelial cell in the context of the bacterium attached to epithelial cells do these proteins induce the various signal transduction changes in the cell that result in the intimate attachment of bacteria and microvilli effacement (Kenny and Finley, 1995). In addition, LEE genes are not only encoded

by EPEC and EHEC 0157:H7 but have been associated with *Hafnia alvei* isolated from children with diarrhea (Albert et al., 1992), and with *Citrobacter rodentium*, an organism that causes murine colonic hyperplasia but not diarrhea (Schauer and Falkow, 1993). Various *E. coli* strains that are capable of formation of the A/E lesion, both Shiga toxin (Stx) positive and Stx negative, have been isolated from diarrheal fecal samples from rabbits (Cantey and Blake, 1977), calves (Fischer et al., 1994), pigs (Zhu et al, 1994) and dogs (Drolet et al, 1994).

F. Enterohemorrhagic *E. coli* (EHEC), a subset of STEC.

I. *Discovery of STEC*

In 1977 Konowalchuk and colleagues recovered 7 clinical isolates of *E. coli* from patients with severe diarrheal illness. Each of these isolates produced a toxin that was lethal to Vero cells *in vitro* (Konowalchuk et al., 1977). These investigators named that toxin verotoxin. Independently, O'Brien et al. reported that certain strains of *E. coli* produced a cytotoxin that was lethal for HeLa cells (O'Brien et al., 1977). Purification and characterization of the toxin by O'Brien and colleagues showed that this *E. coli* toxin had biological properties like that of Shiga toxin produced by *Shigella dysenteriae* type 1, and the toxin was neutralized by anti-Shiga toxin antibodies (O'Brien et al., 1982). The original designation for this *E. coli* toxin described by O'Brien et al. was Shiga-like toxin (SLT); these toxins are now called Shiga toxins (Stxs) and will be so referenced hereafter. In 1983 O'Brien et al. reported that verotoxin and Shiga toxin were the same proteins (O'Brien et al. 1983b) and that this toxin was produced by a novel agent responsible for a food-borne outbreak of bloody diarrhea (Riley et al., 1983), *E. coli* 0157:H7. Later it became clear that the Shiga toxin produced by STEC organisms was responsible for the kidney damage suffered by

several hemolytic uremic syndrome (HUS) patients (Karmali et al, 1983 and Karmali et al, 1985). Furthermore, Karmali noted that *E. coli* 0157:H7 was not the only serotype of STEC that was associated with disease in HUS patients, and he surmised that these non-0157 STEC may play a significant role in the disease.

2. *Geographic distribution of STEC.*

Reports confirm that *E. coli* 0157:H7 is the most frequently isolated cause of hemorrhagic colitis (HC) and HUS disease in the United States, Canada, the United Kingdom and Japan (Reilly, 1998). An unprecedented outbreak in 1996 occurred in Japan with a reported 9,451 cases of STEC infections caused by 0157:H7 (Reilly, 1998). There were 1,808 people (many children) hospitalized and 12 deaths. In the United States, there are an estimated 20,000 infections and 250 deaths attributed to STEC of serotype 0157:H7 annually (Reilly, 1998). However, non-0157 STEC pose a serious threat to the public health as well. For example, 0111:NM and 0104:H21 serotypes caused recent outbreaks in the United States. These outbreaks would not have been discovered if only routine laboratory screening procedures had been used because strains 0111 and 0104 ferment sorbitol and would not have been distinguished from normal flora *E. coli* on sorbitol-MacConkey's agar used to identify sorbitol non-fermenting 0157:H7 isolates (Banatvala, et al., 1996). Non-0157 STEC play a significant role in outbreaks in other parts of the world such as Australia, Argentina, and China (Reilly, 1998). Over 100 non-0157:H7 STEC serotypes have been isolated from patients with HC and several non-0157:H7 serotypes have also caused HUS in infected persons (Willshaw, et al., 1992; Griffin, 1995; Banatvala et al., 1996; Bokete, 1997; Schmidt,

1999; Bonnet et al., 1998;). Some of those serotypes most frequently isolated from human infections are 026, 0103, 0111, 0113 and 0128 (Griffin, 1995).

3. *Clonally divergent subgroups of STEC.*

More than 36 different O:H serotypes have been isolated from humans with disease and more than 60 divergent serotypes have been cultured from cattle and food (reported by Lior, 1994). Clearly, the diversity of *E. coli* isolates that carry *stx* genes is immense. In an effort to group and classify STEC, Whittam and colleagues (Whittam, 1998) used the multilocus enzyme electrophoresis method (Selander et al., 1986) to estimate the genetic relationships among *E. coli* strains that carry the *stx* gene. [The similarity of the electrophoretic mobility of specific alleles of housekeeping enzymes between strains indicates relatedness (Whittam et al. 1993)]. When the data were compiled and analyzed, four major clonally related groups of Stx-producing *E. coli* were defined: EHEC 1, EHEC 2, STEC 1, and STEC 2. Please note that all four groups belong to the large category of STEC.

EHEC 1 is the most common Stx-positive group associated with human disease. EHEC 1 consists of 0157:H7, its sorbitol fermenting derivative, and the non-motile variant (Whittam et al., 1988; Whittam et al., 1993). The only other O type found in this group is 055:H7. All EHEC 1 make intimin and contain the LEE locus. Members of the EHEC 2 group are non-0157 serotypes. Some of the serotypes associated with this group are 0111:H8, 026:H11 along with a variety of other nonmotile or nontypeable (with standard antisera) isolates. Because EHEC 2 group members have the same virulence factors as *E. coli* 0157:H7 (i.e. encode the LEE and make intimin) and are recovered from HC and HUS patient stool samples, they are classified together with 0157:H7 as EHEC. However, evolutionary

genetic analysis indicates that group EHEC 2 is distinct from *E. coli* O157:H7 (Whittam et al. 1998).

Strains of STEC 1 typically do not express intimin nor do they carry the LEE pathogenicity island. STEC 1 members are represented by diverse serotypes but many of the strains express H21 flagella antigen (Whittam, 1998). The most common serotypes of STEC 1 are O113:H21, OX3:H21 and O91:H21. Strain B2F1 is included in the STEC 1 group. Many STEC 1 strains produce a variant of Stx2. Members of this group are commonly isolated from humans with disease and cattle in North America, Europe, and Asia. The fourth group, STEC 2, is composed of three clonally related serotypes O103:H2, O103:H6 and O45:H2 that are also LEE locus and intimin-negative. Clonal analysis indicates that STEC 2 are highly divergent from that of other STEC groups. Many isolates of STEC 2 encode H2 flagella antigen. STEC 2 appear to have a broad host range and a wide geographic distribution. (Whittam, 1998).

4. *Vehicles of transmission of STEC.*

Contaminated food appears to be the major vehicle of STEC transmission. Undercooked ground beef, roast beef, ham, turkey, cheese, potatoes, unpasteurized milk and apple juice, fermented sausage, and raw fruits and vegetables have all been implicated in the transmission of STEC organisms to humans. Water-borne as well as person to person transmission (daycare centers and nursing homes) in sporadic cases or outbreaks have also been reported (Samadpour et al., 1993; Brewster, et al., 1994; Cransberg et al.; 1996 Reilly, 1998). STEC of various serotypes have been isolated from and or detected in the feces and milk of sheep and cattle (Gyles et al., 1998; Meng et al., 1998; Fagan, 1999). This suggests

that herd or food source animals may be an important reservoirs for STEC. Direct contact with farm animals and birds carrying the organism is also a recognized source of infection (Renwick et al., 1993). The role of asymptomatic food handlers in outbreaks is unclear but it may be important because of the low infectious dose needed for transmission (Griffin, 1995; Tuttle et al., 1999).

5. *Illness associated with STEC Infections.*

Individuals infected with STEC manifest clinical symptoms and complications that are both overlapping and distinct from other acute diarrheal illnesses such as those caused by *Campylobacter*, *Salmonella*, and *Shigella spp.* (Slutsker et al., 1997). Symptoms occur within 3-8 days after ingestion of contaminated food or water. Illness starts with crampy abdominal pain, a short-lived fever followed by non-bloody diarrhea. Bloody diarrhea follows within 48 hours in most diagnosed *E.coli* 0157:H7 infections and is usually accompanied by increased abdominal pain and tenderness. At this time, patients are not febrile, and stool samples contain few or no leukocytes. Hemorrhagic colitis or Bloody diarrhea due to *E. coli* 0157:H7 and other STEC, generally last between four to ten days. However, about 5 to 10% of patients with HC progress to the major complication of infection by STEC, HUS (Griffin, 1995). HUS is thought to be caused by Shiga toxin that is absorbed systemically from the intestine and targeted to the vascular endothelial cells of the kidney. *In vitro* studies confirm that Shiga toxin is extremely toxic to vascular endothelial cells of the kidney (Louise and Obrig, 1995). HUS is life threatening in young children and in the elderly. The syndrome is characterized by hemolytic anemia, thrombocytopenia, with progression to renal failure due to glomerular damage (Slutsker et al., 1997; Remuzzi and Ruggenenti, 1998). About 3-5% of

patients with HUS die (Remuzi and Ruggenti, 1998). In 10-30% of survivors, long-term or life-long renal dysfunction occurs (Tarr, 1994; Remuzi and Ruggenti, 1998). Once the toxin has gained access to the systemic circulation, microvascular damage may also develop at other target organs. Such damage may manifest as thrombotic thrombocytopenic purpura (TTP), a systemic form of thrombotic microangiopathy with neurologic manifestations (Remuzi and Ruggenti, 1998) that is sometimes fatal.

6. *Treatment of HUS.*

Prospective analysis of clinical cases indicates that treatment of 0157:H7-infected patients with trimethoprim-sulfamethoxazole or gentamicin is associated with an increased risk of HUS or TTP (Ostroff et al., 1989; Remuzzi and Ruggenti, 1998). In contrast, other retrospective clinical studies do not support this finding. However, *in vitro* studies show that when *E. coli* 0157 is grown in the presence of the antibiotic trimethoprim-sulfamethoxazole, the level of Shiga toxin production increases although it is not known if this phenomenon happens in humans (Karch et al., 1986). Therefore, treatment of STEC infections with antibiotics is not recommended in the United States. Anti-motility agents given to patients for greater than 24 hours increases the risk of *E. coli* 0157 infection progressing to HUS (Cimolai et al, 1992; Cimolai, 1993). Thus, except for supportive care and hemodialysis, no treatment has been shown to decrease the severity of illness. However, specific chimeric-humanized monoclonal antibodies that neutralize the activity of Stx1, Stx2 and variant Stx2d have been developed (Edwards et al., 1998). These chimeric monoclonals protect streptomycin-treated mice fed STEC against the lethal effects of the toxin (Edwards

et al., 1998). In the future, passive therapy with these monoclonals may serve to reduce the severity of illness in STEC-infected patients.

7. *Isolation and detection of STEC organisms.*

Sorbitol-MacConkey media (SMAC) is used extensively to isolate 0157 from patient stool samples and differentiate these organisms from other fecal *E. coli* that ferment sorbitol within 24 hours (Wells et al, 1983; March et al., 1986). When colorless colonies are isolated on SMAC, they are selected and tested with specific O serotype antibody to determine if the isolates are 0157:H7. Test reagents for the serological identification of the 0157 antigen are commercially available (i.e. latex agglutination, Remel, USA). Derivations of the SMAC media have been developed that contain the substrate 4-methylumbelliferyl- β -D-glucuronide (MUG) that when cleaved by β -glucuronidase, fluoresces and releases a product (4-Methylumbelliferone) that is detectable with long wave ultra-violet light (Chapman et al., 1991; Zadik et al., 1993). Because 0157:H7 strains are negative for β -glucuronidase this test discriminates them from other fecal *E. coli* isolates. Only one MUG-positive 0157 EHEC has been detected in the United States, but MUG positive and sorbitol positive strains of 0157 are not uncommon in Central Europe (Gunzer and Karch, 1993; Hays, et al. 1995). *E. coli* 0157:NM (non-motile) strains have been isolated in Europe (Gunzer et al, 1992) and represent the most frequent serotype recovered from stools of HUS patients in Germany. Sorbitol-fermenting 0157:H7 strains have not been detected by North American researchers who have used methods that did not rely on SMAC media to identify clinical isolates as 0157 organisms (reports by Reilly, 1998 and Tarr, 1994).

Shiga toxin has been detected in fecal samples or enrichment cultures by the use of a commercial enzyme immunoassay (EIA) kit (Premier EHEC; Meridian Diagnostics, Inc., Cincinnati, Ohio). This kit is sensitive (detects ≤ 100 pg) and specific but it can not detect a variant of Shiga toxin, Stx2e, produced by *E. coli* 0101:H19 (Allerberger et al., 1996), nor does the kit detect isolates that produce low levels of toxin (Yam et al., 1998).

Standardized, FDA-approved nucleic acid-based assays are not available for routine clinical diagnostic labs to detect STEC but such assays have been used by several reference laboratories around the world to characterize clinical isolates of STEC. Multiplex-polymerase chain reaction (PCR) has also been used to amplify from stool samples, primary fecal cultures, and isolated colonies various target genes that are known to be encoded by Shiga toxin-producing *E. coli* (i.e. *stx*, *eae*, *hlyDABC* [STEC-hemolysin operon]). Recently, several researchers have used this method to determine what virulence genes were encoded by strains isolated from specific clinical cases or outbreaks (Meng et al., 1998; Fagan et al., 1999; Schmidt et al., 1999; Bonnet et al., 1998).

Many non-0157 serotypes have been isolated from clinical cases of STEC-related illnesses and have caused significant outbreaks in the United States and in most of the developed nations of the world. Therefore, it has become critical to develop rapid and reliable tests that can help identify specific organisms responsible for an outbreak. Subtyping STEC strains below the species level has become an integral part of epidemiologic investigations and has been helpful in tracing the source of outbreaks. Pulsed-field gel electrophoresis (PFGE) has been used for this purpose and is the most widely used fingerprinting method for subtyping STEC pathogens. PFGE can be used to determine if isolates recovered from

clinical cases during an outbreak are the same as those recovered from suspected sources of contamination. This approach was used successfully during the 1996 O157:H7 outbreak in Japan to show that two different strains of O157:H7 were responsible for outbreaks in Hiroshima and Sakai (Izumiya, H. et al. 1997).

8. Major virulence properties associated with STEC.

The primary virulence determinants of STEC are the Shiga toxins (described below). In addition, intimin and the LEE genes encoded by EHEC 1 and EHEC 2 members are critical for A/E lesion formation and *in vivo* colonization (McKee et al. 1995). Colonization factors expressed by LEE locus-negative STEC are clearly important in the pathogenesis of these organisms, but the genes that encode these adherence factors have not been cloned. Other determinants encoded on the large ~90 kb plasmid that may contribute to virulence are: STEC-hemolysin, type II secretion system, catalase-peroxidase, and serine protease. These putative virulence factors will be discussed under a separate heading.

a.) Shiga toxins, major virulence factors encoded by STEC.

Shiga toxins represent a family of potent cytotoxins that are produced by some strains of *E. coli*. There are two antigenic types of Shiga toxin (Scotland et al., 1985; Strockbine et al., 1986); they are designated as Shiga toxin type 1 and Shiga toxin type 2 (O'Brien et al., 1992). Structurally, the Stxs consist of 1A:5B non-covalently-linked subunits (Donohue-Rolfe et al., 1984; Donohue-Rolfe et al., 1989). The B polypeptide of Stx is approximately 7.7 kDa and forms a pentamer that uses globotriaosylceramide (Gb₃) as its receptor on eucaryotic cells. The A subunit (32kDa) is nicked by trypsin and reduced to an A₁ portion of ~28kDa and an A₂ peptide of 4kDa. After nicking, the A₁ portion is non-

covalently linked to the B pentamer by the A₂ peptide. The B pentamer of Stx encircles the carboxy terminus of the Stx A subunit (Stein et al., 1992), but the position of the C-terminal portion of the A₂ is not known. The A₁ portion of the molecule contains N-glycosidase activity that causes depurination of a critical residue in the 28S rRNA of 60S ribosomes and leads to inhibition of protein synthesis.

Neutralization studies have shown that Shiga toxins belong to two antigenic groups. Group one consists of Stx1 whose activity is neutralized by antiserum specific to Shiga toxin produced by *Shigella dysenteriae* (O'Brien et al., 1982). Group two Stxs are not neutralized by Stx1 specific antisera (Strockbine et al., 1985). This group includes Stx2 and its antigenic variants Stx2c, Stx2d and Stx2e (Perera et al., 1988; Schmitt et al., 1991). Both groups of Stxs (Stx1 and Stx2) are cytotoxic for Vero (monkey kidney epithelial) and HeLa (human cervical epithelial) cells, enterotoxic for rabbits and lethal for rabbits, mice and other animals. Stx1 and Stx2 are only 55% similar at the amino acid level within the A subunit and only 57% within the B subunits. Stx2 is more potent than Stx1 in several models such as mice (Tesh 1993), human renal endothelial cells (Louise et al., 1995), and intestinal endothelial cells (Jacewica et al., 1999). This difference in toxicity is reflected in streptomycin-treated mice fed STEC strains that make both Stx1 and Stx2, or Stx1 (Wadolowski et al., 1990; Lindgren 1993). Stx2 is responsible for the severe necrotic renal tubular lesions and death of STEC-fed mice. Moreover, epidemiological evidence indicates that EHEC 0157:H7 strains that encode Stx₂ are more frequently associated with HUS than are strains that contain only *stx*₁.

Variants exist among the Stx2 group: Stx2c, Stx2d and Stx2e. The mature B subunits of Stx2c and Stx2d are identical to each other but differ significantly from the Stx2e variants, and these differences may be enough to alter their affinity to receptor or even to change the receptor utilized. There are two amino acid differences within the C terminal end of the A₂ subunits between the Stx2c and the Stx2d toxin. These changes in the A₂ subunit of Stx2d are thought to be responsible for the enhanced toxicity of activatable Stx2d on Vero cells and in the streptomycin-treated mouse model. The exact mechanism responsible for activation of Stx2d is under investigation. However, STEC strains that produce Stx2d (e.g. B2F1) have an oral LD₅₀ of less than 10 colony forming units (CFU). In contrast, STEC strains that produce Stx2 or Stx2c have oral LD₅₀ of approximately 10¹⁰ CFU (Lindgren et al., 1993). Mouse virulence of STEC strains is directly related to the type of toxin produced. It is not known what advantage, if any, the presence of activatable toxin confers on STEC organisms during infection in humans.

The *stx* genes of 0157:H7 are carried on lambda-like bacteriophages and have been transferred into non-toxigenic strains in the laboratory (O'Brien et al., 1984; Newland et al., 1985; Strockbine et al., 1986). However, in some STEC strains the *stx* genes are chromosomally encoded, and this appears to be the case for strain B2F1 that encodes the activatable toxin Stx2d (Lindgren 1993).

b.) LEE pathogenicity island encoded by STEC.

Isolates of both EHEC 1 and EHEC 2 groups encode the LEE locus genes. [The LEE locus was described in detail under enteropathogenic *E. coli* (EPEC)]. The LEE is present in the majority of non-0157:H7 STEC strains implicated in human disease

(reviewed by Nataro and Kaper, 1998), and the complete LEE loci from 0157:H7 strain EDL933 and from EPEC E2348/69 have been sequenced and compared. There are major differences between the EPEC and EHEC LEE pathogenicity islands. The first major difference is that the 0157:H7 LEE contains 43,359 bp in contrast to the 35,624 bp from E2348/69 (Kaper et al., 1998). In strain EDL933, most of the difference in size is due to a 7.5 kb putative prophage with homology to the P4 family of prophages that is inserted at the distal end of the LEE closest to the *selC* locus. The presence and position of the prophage suggests that it may have been involved in mobilization of the LEE into this strain, but the placement of the *att* sites contradict this notion (Perna et al, 1999). The other 41 genes that are present in both LEEs are arranged in exactly the same order and number. The 41 genes, on average, are 93.9% homologous but the amount of divergence is heterogeneous among these 41 genes. For example, the *esc* genes that encode the components of the type III secretion system are highly conserved (Elliot, 1998), whereas genes that encode proteins known to directly interact with the target cells of the host are the most divergent. Examples of divergent genes include *esps*, *eae* and *tir*. The *eae* gene of EHEC is 87.23% identical to that of EPEC; the 3' end is the most divergent (Schmidt et al, 1993; Willshaw et al., 1993) and this is the end of the gene that encodes the putative receptor-binding domain of intimin. The *tir* gene is the most divergent of the LEE genes. The Tir protein, produced by EPEC strains, is phosphorylated when it is inserted into the host membrane. Post-translocation phosphorylation does not occur in EHEC 0157:H7 strains that encode *tir* but does occur in EHEC 026:H⁻ (member of EHEC group 2) isolates (Deibel et al.1998). The reason for the phenotypic differences between the prototypic EHEC 0157:H7 and EPEC E2348/69 is not clear.

c.) Adherence of STEC groups 1 and 2 that do not encode the LEE: the subject of this project.

STEC groups 1 and 2 do not encode the LEE pathogenicity island but many of these strains have not only been isolated from animal and food sources (Johnson et al., 1996) but also from patients with HUS and HC (Rilley, 1998). For example, an STEC group 1 serotype 091:H21 strain B2F1 was isolated from a 3 year old with HUS (Dr. M. Karmali, Hospital for Sick Children, Toronto, Ontario). More recently (1996), an 091:H21 strain was isolated from a HUS patient in Clermont-Ferrand, France (Bonnet, et al., 1998). In addition, an 0104:H21 isolate of STEC 1 caused a milk-borne outbreak of HC in Montana in 1994 (Strockbine et al., 1997; Slutsker et al., 1997). These findings taken together lend credence to the hypothesis that STECs can and do adhere to human intestinal epithelial cells. Indeed, in the current study the capacity of 091:H21 B2F1 to adhere to human colonic epithelial T84 cells was demonstrated. The experimental data available on the factors (independent of intimin) necessary for adherence of STEC are summarized below.

Type 1 pili are produced by many *E. coli* species and may play a key role in commensal host-bacterial interactions. Such pili also appear to be a virulence factor in urinary tract infections (Schaeffer et al, 1987; Connel et al., 1996). Type 1 fimbriae mediate attachment to mannose-containing receptors allowing colonization of many host surfaces (Brinton, 1959; Keith et al., 1986; Sokurenko et al., 1994). Specific type 1 pili-dependent adherence of some species of *E. coli* is inhibited in the presence of D-mannose (Ashkenazi et al., 1991). Investigators have explored the role of type 1 pili-D-mannose-sensitive-adherence of STEC (Durno et al., 1989; Winsor et al., 1992) and concluded that type one pili

are not necessary for attachment of STEC to epithelial cells. Indeed, in this study we demonstrated that 091:H21 adherence to T84 cells is not blocked by mannose.

9. *The ~90 kb plasmid encoded by E. coli 0157:H7 and other STEC and its putative role in pathogenesis.*

STEC carry a large plasmid that is ~90 kb in size [the size may vary slightly among strains (Bopp et al., 1987)]. The role of the ~90 kb plasmid in the adherence of prototypic 0157:H7 strains was assessed by several investigators. One study showed that 0157:H7 strains that possessed the large plasmid (~90 kb) elaborated fimbriae and adhered to Henle 407 intestinal cells, whereas the plasmid-cured derivatives failed to express fimbriae or to adhere to intestinal cells (Karch et al., 1987). Another group reported that a plasmid-cured derivative of 0157:H7 showed decreased adherence to intestinal cells and to HEp-2 cells, but full adherence was restored when the plasmid was transformed back into the strain (Toth et al., 1990). In 1993 Fratamico and colleagues reported that *E. coli* 0157:H7 with the plasmid and its plasmidless derivative adhered equally well to HEp-2 cells and to Henle 407 cells. In addition, sparse fimbriae were noted on the surfaces of 0157:H7 with and without the plasmid (Fratamico et al., 1993). Although plasmid p0157, carried by STEC 0157:H7 strain EDL933, has been the subject of numerous investigations, published data in support of a correlation between the presence of plasmid p0157 and adherence of EDL933 to epithelial cells has been conflicting. All of the published information gathered to date concerning the large plasmid encoded by STEC organisms was derived from the study of the large plasmid encoded by strains of *E. coli* 0157:H7 and none of these experiments dealt with STEC *eae* negative strains. In this study, to address the role of the large plasmid encoded by B2F1 in

adherence of this strain to T84 cells, a plasmid cured derivative of B2F1, S11, was assessed for its capacity to adhere to human colonic T84 cells.

The streptomycin-treated mouse model was used to show that growth in mucus and colonization of the small and large intestine were not affected in the plasmid-cured derivative of EDL933. But, when the plasmid-cured derivative (EDL933-cu) and the parent EDL933 strain were fed simultaneously, EDL933-cu was unable to maintain a stable level of colonization in about two-thirds of the mice tested (Wadolkowski et al. 1990). Other investigators proved that plasmid p0157 from strain 933 was not needed to cause the A/E lesion or for manifestation of disease in gnotobiotic piglets (Tzipori et al., 1987).

a.) Description of the ~90 kb plasmid

A physical map of plasmid p0157, isolated from *E. coli* 0157:H7 strain EDL933, was constructed and the plasmid size was determined to be ~90 kb (Schmidt et al., 1996). Two years following the 1996 EHEC outbreak in Japan, the ~90 kb plasmid from a clinical isolate 0157:H7 strain, RIMD 0509952, was sequenced (Makino et al., 1998). Computer analysis of the sequence data identified 84 potential protein coding open-reading-frames (Makino et al., 1998), and several of the ORFs did not match any known nucleotide sequences in the database. However, some of the genes have been cloned by independent investigators and found to encode some putative virulence determinants, and these will be discussed below.

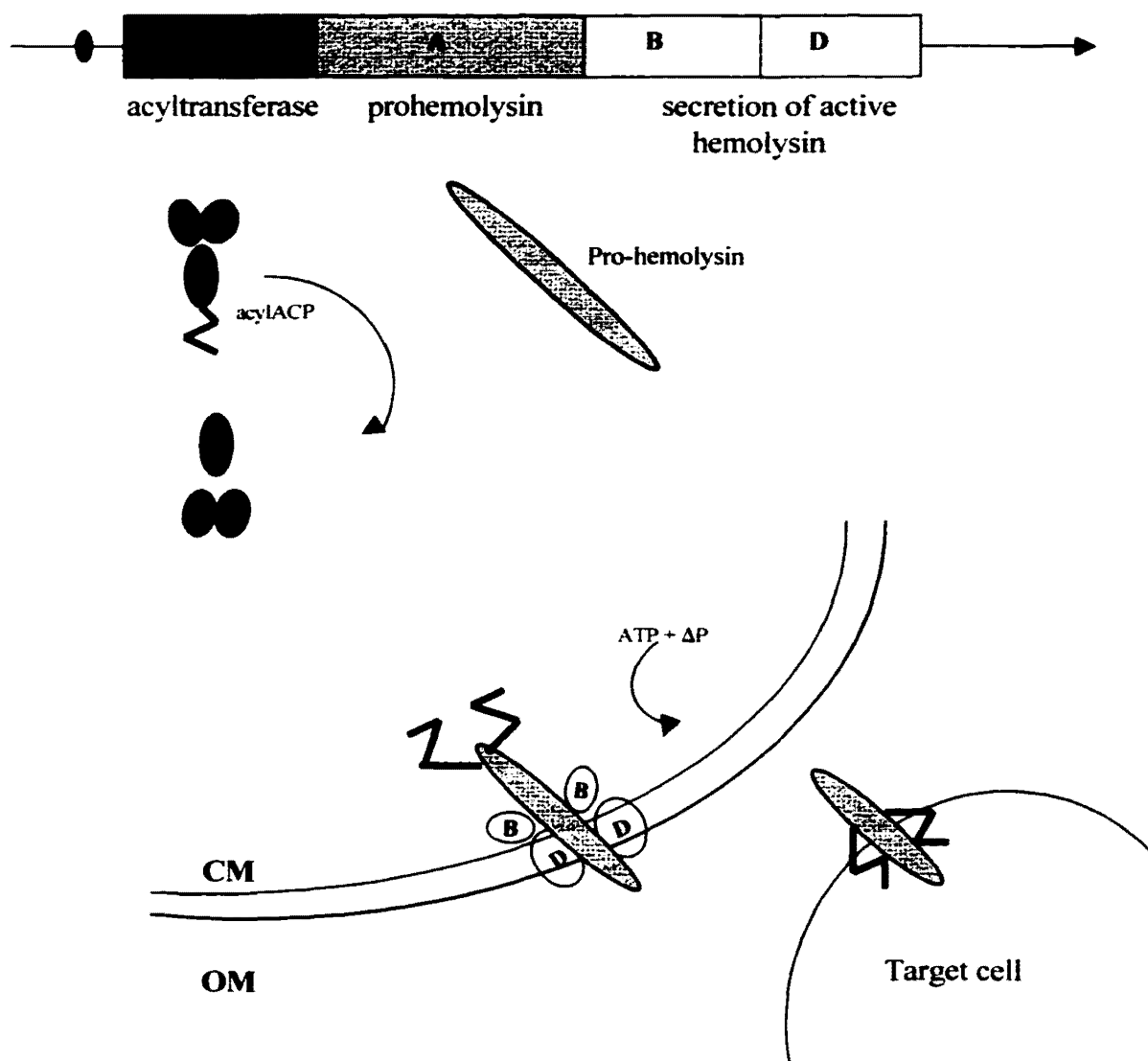
b.) Hemolysin expressed by STEC.

In this study, an H-NS knockout mutant exhibited derepression of hemolysis production. Therefore, I will provide more information on hemolysin than on the

other plasmid-encoded determinants. Please note: The hemolysin encoded on the large plasmid of STEC is hereafter generically called the STEC-hemolysin unless reference is made to results of a specific study of *E. coli* 0157:H7 hemolysin.

The ~90 kb plasmid of *E. coli* 0157:H7 strains encodes hemolysins that are members of the *E. coli* α -hemolysin family (Schmidt et al., 1994). As indicated by nucleotide sequence analysis, the STEC-*hlyA* gene from *E. coli* 0157:H7 strain EDL933 is 61% homologous to the *hlyA* gene of α -hemolysin (Schmidt et al., 1995). Each of the four genes within the operon that encodes the pO157-encoded hemolysin is highly related to the genes of the *E. coli* α -hemolysin operon and other Repeats in Toxin (RTX) cytolytins (Schmidt et al., 1995). Like *E. coli* α -hemolysin, hemolysin genes of EDL933 are organized in an operon structure in the order of *hly*-CABD [Figure 2] (Welch, 1988; Schmidt et al., 1995). The *E. coli* 0157:H7- *hlyA* gene encodes the 107 kDa structural protein of hemolysin. STEC hemolysin requires posttranslational acylation for activity. This modification is done by the *hlyC* gene product, and occurs before the 107 kDa protein is translocated across the bacterial inner membrane. The HlyB and HlyD proteins serve as the translocator unit for the functional hemolysin. TolC (an outer membrane protein) encoded by a gene separated from the α -*hly*-CABD operon cooperates with the HlyB and HlyD protein to translocate the HlyA protein (Wandersman, C. et al., 1990). One difference between wild-type *E. coli* strains that express the STEC-hemolysin and *E. coli* strains that express α -hemolysin (such as uropathogenic strains of J96) is that only small turbid zones of hemolysis are evident around colonies of the EDL933, whereas large clear zones of hemolysis occur around J96.

Figure 2. The STEC-hemolysin operon including a representation of maturation and export by *E. coli*. (CM, cytoplasmic membrane; OM, outer membrane; ΔP , total proton-motive-force). The STEC-*hlyA* gene encodes inactive prohemolysin, which is activated by the acyltransferase, HlyC. HlyA is secreted by a type I process and molecules HlyB and HlyD serve as the transporter of active hemolysin. The genes of the STEC-*hly* operon are represented by capital letters; CABD. Jagged lines attached to hemolysin represent the acylated-state of STEC-hemolysin. Acyl-ACP stands for acyl donor. This figure was modified from a review by Stanley et al. (Stanley et al., 1998).



The α -hemolysin is an important virulence factor in *E. coli* extraintestinal infections such as those of the upper urinary tract (Welch et al., 1981). This toxin is a member of a family of membrane-targeted toxins assumed or proven to play a role in the pathogenesis of not only urinary tract infections but also juvenile periodontitis, pneumonia, whooping cough, and wound infections. Toxins within the family include the leukotoxin of *Pasteurella haemolytica* (Strathdee and Lo, 1987), the bifunctional adenylate cyclase-hemolysin of *Bordetella pertussis* (Glaser et al., 1988), and the hemolysins of *Proteus vulgaris* (Welch, 1987). These toxins share (i) posttranslational maturation, (ii) a C-terminal calcium-binding domain of acidic glycine-nonapeptide repeats that has led to the RTX (repeat toxin) family nomenclature; and, (iii) export out of the cell by type 1 secretion systems (Stanley et al., 1998).

Like α -hemolysin, the *E. coli* 0157:H7-hemolysin protein contains 13 tandem repeats at the C-terminus. Calcium is bound following export of the protein with two or more molecules of calcium per repeat and binding is a requirement for cytotoxic activity (Bauer et al., 1996). The α -hemolysin exhibits little target cell specificity but has a wide spectrum of cytotoxic activity; attacking erythrocytes, granulocytes, monocytes, endothelial cells, and renal epithelial cells of mice, ruminants, and primates (Mobley et al., 1990; Keane et al., 1987; Stanley et al., 1998). Unlike α -hemolysin, the *E. coli* 0157:H7-hemolysin has virtually no lytic activity against human leukocytes (Bauer and Welch, 1996). It is believed (based on experiments that sought to show saturable binding) that receptor-mediated binding is not essential for the hemolysin to intoxicate target cells (Soloaga et al., 1999).

The precise role, if any, that STEC-hemolysin plays in the pathogenesis of disease remains to be determined. Antibody responses against STEC-hemolysin have been detected in the sera of patients recovering from HUS (Schmidt et al., 1995), an indication that hemolysin is expressed during infection. However, not all Shiga toxin-producing *E. coli* produce this hemolysin (Schmidt et al., 1999).

c.) *E. coli* 0157:H7 type II secretion system.

A thirteen gene cluster designated as *E. coli* 0157:H7 type II secretion pathway (*etpC*-O) is upstream of the STEC-hemolysin operon. Sequence analysis indicates that the genes encode a superfamily of proteins involved in various secretion pathways of gram-negative bacteria (Schmidt et al., 1997). The proteins secreted by this putative transport system have not been isolated or characterized.

d.) Bifunctional catalase-peroxidase (KatP).

A gene (*katP*) that encodes a bifunctional catalase-peroxidase is present on the plasmid p0157 of strain 0157:H7 (Brunner et al., 1996). This protein has significant similarity to the catalase-peroxidase genes encoded by *perA* of *Bacillus stearothermophilus*, and the chromosomally encoded HPI catalase KatG of *E. coli*. The protein produced by *katP* is present in wild-type *E. coli* 0157:H7, mainly in the periplasm. This protein functions as a catalase and a peroxidase.

e.) Serine protease (EspP).

The *espP* gene produces an extracellular serine protease that cleaves human coagulation factor V (Brunner et al., 1997). It is possible that cleavage of this critical blood clotting factor may contribute to the mucosal hemorrhage observed in patients with

hemorrhagic colitis (Makino et al., 1998). Homologues of EspP exist in EPEC (EspC), *Shigella flexneri* (SepA), and in an avian-pathogenic *E. coli* (Tsh) [Stein et al., 1996; Benjelloun-Touimi et al., 1995; Provence et al., 1994]. The protease is synthesized as a large precursor and processed extensively at the amino-terminus and carboxy-terminus during secretion to yield the mature active form of the protein (104 kDa). Secretion of *espP* is regulated by both temperature and pH and expression is optimal when bacteria are grown at pH 7 at 37° C. Since convalescent sera contain antibodies to EspP, the protease is probably expressed at some time during infection (Brunder et al., 1997).

f.) Distribution of the plasmid-encoded putative virulence traits among STEC.

In a recent study, the distribution of plasmid encoded determinants among STEC isolates was analyzed (Karch et al, 1998). A total of 50 *E. coli* 0157 and 50 non-0157 clinical isolates were evaluated by multiplex-PCR. Also, 30 strains each of ETEC, EaggEC, EPEC and EIEC bacteria were included as controls. The STEC-*hlyA* gene and the type II secretion gene (*etpO-C*) occurred in almost all the EHEC 0157 isolates but the *katP* and *espP* genes were present in only 66%. The most prevalent marker in non-0157 isolates was STEC-*hlyA* (95%) and *etpO-C*, *katP* and *espP* were encoded in 52%, 38% and 36% of the strains, respectively (Karch et al, 1998). All other strains of enteric *E. coli* pathogens were negative for these markers.

g.) Description of probe CVD419.

Levine et al. developed a probe (CVD419) for the detection of the large plasmid in STEC. The probe consists of a 3.4 kb *HindIII* fragment of plasmid p0157 that is

carried by *E. coli* EDL933 (Levine et al., 1987). The 3.4 kb fragment contains a major portion of the *hlyA* gene and part of the *hlyB* gene (Schmidt et al., 1995). The probe has been used successfully in the past to detect 0157:H7 strains but now it is used infrequently for detection of other STEC.

IV. The histone-like-nucleoid-structuring protein (H-NS).

G. Discovery of H-NS and the types of systems regulated by H-NS.

The *hns* gene was initially cloned from *E. coli* by Pon et al. in 1988. The gene encodes a protein that consists of 137 amino acids (15.4 kDa) [Figure 3]. H-NS was originally shown to be involved in the organization of the bacterial chromosome by modulation of the level of DNA condensation, much like the eukaryotic histones (Spassky et al., 1984); thus, the name H-NS, histone-like nucleoid structuring protein. H-NS is a major component of the *Escherichia coli* nucleoid (~20,000 copies/cell) and is, in fact, one of the most abundant DNA binding proteins in Enterobacteriaceae.

H-NS is a global modulator of a variety of unlinked and unrelated genes in *E. coli* and *Salmonella typhimurium* (Ussery et al., 1994; Atlung and Ingmer, 1997; Dorman et al. 1999). Mutations in *hns* are pleiotropic and affect the synthesis, both positively and negatively, of more than 50 *E. coli* proteins (Bertin et al., 1990; Yamada et al., 1991; Laurent-Winter et al., 1995; Laurent-Winter et al., 1997) including H-NS itself (Free and Dorman, 1995). Many

Figure 3. Multiple sequence alignment of H-NS homologues. The numbers refer to the residues from the *Escherichia coli* H-NS amino acid sequence. Residues in boxes represent non-identity between residues. The consensus sequence is indicated below the aligned sequences. The sequences aligned are: H-NS *E. coli*, Sw:P08936; H-NS *Shigella flexneri*, Sw:P09120; H-NS *Salmonella typhimurium*, Sw:P17428; and StpA *E. coli*, Sw:P30017. Sw refers to the Swissprotein databases. The oligomerization domain, the linker region, the nucleic-acid-binding domain, and the region responsible for protein-protein interactions are indicated. This figure was modified from a publication by Dorman and colleagues (Dorman et al., 1999).

	Oligomerization domain																																											
								10								20								30								40												
H-NS <i>E. coli</i>	M	S	E	A	L	K	I	L	N	N	I	R	T	L	R	A	Q	A	R	E	C	T	L	E	T	L	E	E	M	L	E	K	L	E	V	V	N	E	R	R	E	E	E	
H-NS <i>S. flexneri</i>	M	S	E	A	L	K	I	L	N	N	I	R	T	L	R	A	Q	A	R	E	C	T	L	E	T	L	E	E	M	L	E	K	L	E	V	V	N	E	R	R	E	E	E	
H-NS <i>S. typhimurium</i>	M	S	E	A	L	K	I	L	N	N	I	R	T	L	R	A	Q	A	R	E	C	T	L	E	T	L	E	E	M	L	E	K	L	E	V	V	N	E	R	R	E	E	E	
StpA <i>E. coli</i>	M	S	V	M	L	Q	S	L	N	N	I	R	T	L	R	A	M	A	R	E	F	S	I	D	V	L	E	E	M	L	E	K	F	R	V	V	T	K	E	R	R	E	E	E
consensus	M	S	E		L	K	I	L	N	N	I	R	T	L	R	A	Q	A	R	E	C	T	L	E	T	L	E	E	M	L	E	K	L	E	V	V	N	E	R	R	E	E	E	

	Oligomerization domain															Linker region															Protein-protein interactions (67-137)														
	50					60					70					80																													
H-NS <i>E. coli</i>	S	A	A	A	E	V	E	R	T	R	K	L	Q	Q	Y	R	E	M	L	I	A	D	G	I	D	P	N	E	L	L	N	S	L	A	A	V	K	S	G	T	K	A			
H-NS <i>S. flexneri</i>	S	A	A	A	E	V	E	R	T	R	K	L	Q	Q	Y	R	E	M	L	I	A	D	G	I	D	P	N	E	L	L	N	S	L	A	A	V	K	S	G	T	K	A			
H-NS <i>S. typhimurium</i>	S	A	A	A	E	V	E	R	T	R	K	L	Q	Q	Y	R	E	M	L	I	A	D	G	I	D	P	N	E	L	L	N	S	L	A	A	V	K	S	G	T	K	A			
StpA <i>E. coli</i>	E	Q	Q	Q	R	E	L	A	E	R	Q	E	K	I	S	T	W	L	E	L	M	K	A	D	G	I	N	F	E	E	L	G	N	S	S	A	A	P	R	A	G	K			
consensus	A	A	A	E	E	R	T	R	K	L	Q	Q	Y	R	E	M	L	I	A	D	G	I	D	P	N	E	L	L	N	S	L	A	A	V	K	S	G	T	K	A					

	Nucleic acid-binding domain															Protein-protein interactions																																
	90	100	110	120	130																																											
H-NS <i>E. coli</i>	K	R	A	Q	R	P	A	K	Y	S	Y	D	E	N	G	E	T	K	T	W	T	G	Q	G	R	T	P	A	V	I	K	K	A	M	D	E	Q	G	K	S	L	D	D	F	L	I	K	Q
H-NS <i>S. flexneri</i>	K	R	A	Q	R	P	A	K	Y	S	Y	D	E	N	G	E	T	K	T	W	T	G	Q	G	R	T	P	A	V	I	K	K	A	M	D	E	Q	G	K	S	L	D	D	F	L	I	K	Q
H-NS <i>S. typhimurium</i>	K	R	A	Q	R	P	A	K	Y	S	Y	D	E	N	G	E	T	K	T	W	T	G	Q	G	R	T	P	A	V	I	K	K	A	M	D	E	Q	G	K	S	L	D	D	F	L	I	K	Q
StpA <i>E. coli</i>	K	R	Q	P	R	P	A	K	Y	K	F	T	D	Y	N	G	E	T	K	T	W	T	G	Q	G	R	T	P	K	F	I	A	Q	A	L	A	E	G	K	S	L	D	D	F	L	I		
consensus	K	R				R	P	A	K	Y			D	N	G	E	T	K	T	W	T	G	Q	G	R	T	P	I	A						G	K	S	L	D	D	F	L	I					

target genes that are negatively affected by H-NS are regulated by environmental factors such as osmolarity, temperature, oxygen tension, pH or growth phase. The expression of genes that code for certain pili and virulence factors is thermoregulated (i.e. Pap pili and VirF); expressed at 37°C but not at 26-30°C. Typically, H-NS represses transcription of genes in response to environmental cues, and mutations in H-NS cause derepression of these genes (Table 1). Examples of systems in which H-NS negatively regulates gene expression include: *proU*-osmotic regulation (Higgins et al., 1988; Hinton et al., 1990; May et al., 1990), *fimB*-type 1 pili phase variation (Donato et al., 1997), *bgl*- β -glucosidase (Defez and DeFelice, 1981) and *pap*-thermoregulation of Pap pili (Göransson et al., 1990).

In addition, a number of H-NS-negatively-modulated genes are also regulated by positive transcription factors, such as VirB that transactivates the *ipa*, *mxi* and *spa* virulence operons of enteroinvasive *E. coli* in response to increased concentrations of its positive regulator VirF (Dagberg et al., 1992). For example, in the wild-type EIEC background the expression of *virB* at 30°C is reduced, and invasion virulence genes are not expressed. The VirF protein activates the expression of *virB* at 37°C to turn on invasion genes in the wild-type background. In contrast, in a *hns* null mutant of EIEC, invasion genes are expressed inappropriately at 30°C because *virF* expression is derepressed due to the absence of H-NS.

H-NS also activates the expression of some genes. For example, evidence suggests that *hns* is a positive regulator of *flhCD*, the master regulon responsible for flagellar biogenesis. In fact, data indicate that null mutations in *hns* abolish motility in *Salmonella*

Footnotes for Table 1

^a Presence of bend in DNA within the promoter region: +, experimentally determined; (+), predicted bend; (-) no predicted bend according to annotations from GeneBank; ND not determined.

^b Experimentally determined *in vitro* by gel retardation and/or footprinting.

^c Expression in *hns* null or *hns* wild-type background calculated from data determined with reporter gene fusions or from direct transcript measurements. Expression determined in the presence or absence of inducing stimuli and/or inducing factors.

This table was compiled from a review paper published by Atlung et al. (Atlung et al, 1997).

Table 1. Examples of genes repressed by H-NS.

Gene	Function	Bent ^a DNA	H-NS binding ^b	Additional regulation ^c		Reference
				inducing stimuli	factor(s)	
<i>adi</i>	Arginine decarboxylase	(-)	ND	Low pH	Supercoiling	Shi et. al, 1993
<i>bolA</i>	Morphogene; affects cell division and growth	+	ND	Stationary phase	RpoS	Barth et al., 1995
<i>bgl</i>	β -glucosidase	+	ND	Not known	CRP, BglC	Higgins et al., 1988
<i>cadBA</i>	Lysine decarboxylase	(-)	ND	Low pH	CadC	Shi et al., 1993
<i>cbpA</i>	DnaJ homologue	+	ND	Low phosphate stationary phase	RpoS	Yamashino et al., 1994; 1995
<i>csgA</i>	Curli subunit	ND	ND	Low temperature; low osmolarity	RpoS	Arnqvist et al., 1994
<i>cfaAB</i>	CFA/I pili	ND	ND	37°C	CfaD	Jordi et al., 1992
<i>fimB</i>	Recombinases for type 1 pili	-	+	37°C	Lrp	Kawula et al., 1991; Donato et al., 1999

Table 1. Continued

Gene	Function	Bent DNA	H-NS binding	Additional regulation		Reference
				inducing stimuli	factor(s)	
<i>hns</i>	Global regulator; DNA binding protein	+	+	Cold shock	Fis, CspA	Atlung et al., 1997
<i>lrp</i>	DNA binding protein	ND	+	Growth phase	Lrp	Oshima et al., 1995
<i>ompC</i>	Outer membrane protein	(+)	+	High osmolarity;	OmpR, Lrp	Suzuki et al., 1996
<i>osmC</i>	Periplasmic protein	ND	ND	High osmolarity stationary phase	Lrp, CRP, RpoS	Barth et al., 1995
<i>papBA</i>	P pili	ND	+	37°C	Lrp	White-Ziegler et al., 1998
<i>rrnB</i>	Ribosomal RNA	+	+	Not known	Fis, ppGpp	Tippner et al., 1994
<i>stpA</i>	RNA chaperone	ND	+	No leucine	StpA, Lrp	Zhang et al., 1996; Sonden et al., 1996
<i>virB_{Sh}</i>	activator of invasion genes	+	+	37°C	VirF	Tobe et al., 1993

typhimurium (Hinton et al., 1992) and in *E.coli* (Bertin et al., 1994).

B. Proposed mechanisms by which H-NS modulates gene expression.

In vitro studies revealed that at physiological concentrations (concentrations of H-NS normally found in the bacterial cell), H-NS forms homodimers and binds to intrinsically curved DNA sequences located near prokaryotic promoters (Tanaka et al., 1991). DNA *in vitro* footprinting and gel-retardation studies have shown that H-NS normally protects large regions of DNA and that multiple H-NS molecules bind co-operatively (Lucht et al., 1994; Tippner et al., 1994). H-NS itself can bend DNA, and this activity may explain how H-NS interacts with DNA that is not naturally curved (Spurio et al., 1997). H-NS also binds non-specifically to DNA in a sequence-independent manner (Tupper et al., 1995; Lucht et al., 1994). Nevertheless, the exact mechanism by which H-NS affects transcription is not clear for most of the genes that it regulates. One hypothesis is that H-NS affects transcription of a gene by changing the supercoiling of the DNA, which in turn, modulates promoter activity (Hinton et al., 1988). Alternately, H-NS may bind to the promoter region of a gene and interfere with RNA-polymerase interacting productively with a promoter, a possibility suggested by the findings on H-NS-mediated repression of *E. coli proU* and EIEC *virB* (Ueguchi and Mizuno, 1993; Colonna et al., 1995). Such a scenario is inadequate to explain the H-NS-mediated repression of *E. coli hns* and *Salmonella typhimurium proU*, because the H-NS binding sites are located outside the promoter sequences of these genes. Recently, H-NS was also shown to bind avidly to multiple sites within the *fimB* promoter element (Donato et al., 1997) and regulate expression of that gene. Another proposed mechanism by which H-NS negatively affects gene transcription

is that it 'silences' a DNA region (Goransson et al., 1990) by constraining negative supercoils (Tupper et al., 1994).

C. Structure-function analyses of H-NS.

Mutational analyses have demonstrated that H-NS has a two-domain structure comprised of a carboxy-terminal DNA-binding domain (Shindo et al., 1995) and an amino-terminal oligomerization region (Ueguchi et al., 1997; Williams et al., 1996). These two domains are linked by a short (residues 76-88) protease-sensitive (exposed) domain (Dorman et al., 1999). The DNA-binding domain of H-NS is unique in that its three-dimensional structure does not show any similarity (no zinc-finger motif, etc.) to other known DNA-binding proteins (Shindo et al., 1995). Specific mutations such as amino acid deletions or exchanges within the carboxy-terminal domain of H-NS affect its capacity to bind to DNA. Mutations within the carboxy-terminal domain of H-NS between amino acid residues 90 and 120 reduce binding of H-NS to the *fimB* promoter (Donato et al., 1998). Indeed, a change of tryptophan residue 108 to isoleucine renders the H-NS mutant completely unable to bind to the *fimB* promoter (Donato et al., 1999). Furthermore, analysis of deletion mutants indicates that the core DNA-binding motif (i.e. TWTG-GR-P) lies between residues 108 and 116 and is highly conserved among gram-negative bacteria that encode H-NS and H-NS-like proteins (Figure 3).

Recently, multimerization has been shown to be critical for H-NS repressor function at both the *proU* and *hns* promoter regions (Spurio et al., 1997; Ueguchi et al., 1997). Analysis of mutants defective in oligomerization indicates that the multimerization region lies between amino acids 15 to 70 of the H-NS protein and that this region is predicted to form a coiled-

coiled structure ((Ueguchi et al., 1996; Dorman et al., 1999). In addition, this small amino-terminal portion of H-NS is able to form homodimers and to form mixed dimers with intact H-NS *in vivo* (Williams et al., 1996). However, mixtures of truncated and wild-type H-NS dimers are not able to regulate the expression of *proU* (Williams et al., 1996). This dominant-negative mutant phenotype demonstrates that H-NS interacts with DNA as an oligomer and that the carboxy-terminal portion of the molecule is important in regulation of gene expression.

In 1994 Bertin et al., showed that *hns* null mutants of *E. coli* K-12 were non-motile and that H-NS was a positive regulator of the *flhCD* master operon that controls the biogenesis of flagella in *E. coli*. Donato and Kawula (1999) reported that *hns* mutants that contained a substitution of the tryptophan residue 108 for isoleucine were hypermotile. Since tryptophan residue 108 had been previously shown to be critical for H-NS-DNA binding, the results indicate that H-NS-DNA binding was not required to regulate flagella biogenesis in *E. coli* K-12. By contrast, truncation of amino acids at the carboxy-terminus of H-NS resulted in reduced or completely nonmotile mutants. Donato and Kawula concluded that H-NS may also regulate gene expression by protein-protein interactions, and that conformation of the carboxy-terminus of H-NS may be important in these protein-protein interactions.

H-NS exists as three isoforms that differ in their pI values (Spassky et al., 1984; Ussery et al., 1994; Laurent-Winter et al., 1995; Donato et al., 1999). The biological function of each isoform is still unknown. However, the region between amino acids 18 to 26 appears to be the site of posttranslational modification that yields the three isoforms (Donato et al., 1999).

D. Comparison of the structure and function of H-NS and H-NS-like proteins encoded by other gram-negative bacteria.

H-NS exhibits strong amino acid conservation among the Enterobacteriaceae (Atlung et al., 1997) as well as in related bacteria such as *Erwinia chrysanthemi* and *Haemophilus influenzae* (Bertin et al., 1999). Moreover, StpA, a H-NS-like protein with 57% identity with H-NS (Figure 3) has been isolated in *E. coli* (Zhang et al., 1996). Protein cross-linking and genetic studies indicate that H-NS and StpA can form heteromeric complexes *in vivo*. Indeed, data from two-dimensional gel analysis of total proteins in *E. coli* show that certain genes require both proteins for normal regulation (Dorman et al., 1999). BpH3, from *Bordetella pertussis* exhibits amino acid conservation with H-NS, especially at the C terminus. A gram-negative bacterium that is phylogenetically distant from *E. coli*, *Rhodobacter capsulatus*, encodes a transactivator (HvrA) that has a low but significant similarity with H-NS and other H-NS-like proteins (Bertin et al., 1999). Interestingly, mutations in H-NS that affected such characteristics as motility were able to be complemented partially or completely by StpA, BpH3 and other H-NS-like proteins (Bertin et al., 1999). Sequence conservation and distribution of hydrophobic clusters suggest that the secondary and three-dimensional structures of the C domains of these proteins, H-NS, StpA, BpH3 and HvrA are similar. Structural topology of the C-terminal domain is conserved; the hydrophobic core of H-NS that holds this structure together is built around the interactions of residues W108 and Y97, Y99, F134 and I135.

V. Specific Aim

The main goal of my research project was to identify bacterial factor(s) important for the adherence of intimin-negative STEC strain B2F1 to human colonic epithelial cells. The following steps were taken in an attempt to achieve this goal. First, an existing adherence assay was modified to assess the capacity of strain B2F1 to bind to the human colonic epithelial cell line T84. Second, transposon mutagenesis of B2F1 was undertaken to isolate non-adherent mutants of B2F1. Third, mini-Tn5*phoA*Cm^r nonadherent mutants of B2F1 were further characterized. Although I showed that *hns* regulates both adherence and STEC-hemolysin production in B2F1, I was not able to identify specific adhesins of this organism.

Materials and Methods

Cell lines, bacterial strains, and plasmids. Two cell lines were used in these studies: T84 cells that are of human colonic epithelial cell origin (CCL248) and HEp-2 cells that are derived from human laryngeal epithelial cells (CCL23). Both cell lines were obtained from the American Type Culture Collection, Manassas, VA. T84 cell cultures were maintained by serial passage in a 1:1 mixture of Dulbecco's Eagles Minimal Essential Medium (DMEM) and Ham's F12 medium (Biowhittaker, Walkersville, MD) supplemented with 5% fetal calf serum, 20mM L-glutamine, 100 µg/ml gentamicin, 100 Units of penicillin G, and 0.4% sodium carbonate. HEp-2 cells were grown in Eagles Minimal Essential Medium (EMEM) with 10% fetal calf serum, 100 µg/ml gentamicin, 100 U penicillin G, and 0.4% sodium bicarbonate. The *E. coli* strains and plasmids used in this study are described in Tables 2 and 3, respectively.

Media, enzymes, and biochemicals. Bacterial strains were routinely cultured in Luria-Bertani (LB) broth or agar (Difco Laboratories, Detroit, Mich), or on MacConkey agar (Difco), or M-9 minimal agar (Maniatis et al., 1989), or Maloney agar (Maloney, 1972). Tryptose Blood Agar Base (Difco) supplemented with 5% defibrinated sheep blood (Cocalico) was used to assess the hemolytic phenotypes of wild-type 091:H21 strain B2F1 and various mini-Tn5*phoACm'* mutants of B2F1. Where indicated, antibiotics (USB, Cleveland, Ohio) were used to supplement various cultures. The concentrations of antibiotics used to supplement

cultures were as follows: 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, 50 µg/ml kanamycin, 50 µg/ml nalidixic acid, 12.5 µg/ml tetracycline, and 30 µg/ml streptomycin.

Recombinant plasmids with DNA inserts that inactivated *lacZ* were detected by screening *E. coli* K-12 transformed with the plasmids for white colonies in the presence of 20 µg/ml of the colorimetric substrate, X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactoside (Sigma Chemical Co., St. Louis, MO)]. Mutants of B2F1 that contained mini-Tn5*phoA*Cm^r insertions were selected as blue (PhoA⁺) or white (PhoA⁻) colonies on agar that contained the substrate for alkaline phosphatase (Sigma), 5-bromo-4-chloro-3-indolyl phosphate abbreviated as XP, and chloramphenicol.

Restriction endonucleases were purchased from New England BioLabs (Beverly, MA). T4 DNA ligase kits were obtained from Invitrogen, Carlsbad, CA. Enzymes and kits were used according to the specific manufacturers' instructions. The ECLTM nucleic acid detection kit (Amersham Lifescience, Buckinghamshire, England) was used to visualize DNA-DNA hybridization reactions.

Table 2. *E. coli* strains used in this study

Strain	Relevant characteristics	References
STEC strains		
B2F1	O91:H21 serotype <i>eae</i> negative, hemolysin positive; human clinical isolate	Karmali et al., 1985
S11	~90 kb plasmid-cured derivative of B2F1; hemolysin negative	obtained from Dr. A. Melton-Celsa
86-24	O157:H7 serotype, <i>eae</i> positive; human clinical isolate	obtained from Dr. P. Tarr
86-24 <i>eae</i> Δ10	<i>eae</i> deletion mutant of 86-24	McKee et al., 1995
H30	O26:H11 serotype, <i>eae</i> positive; human clinical isolate	Konowalchuk et al., 1977
S11366.15	<i>hns</i> ::pMS358 derivative of S11; hemolysin negative	this study
933	O157:H7 serotype, <i>eae</i> positive, human clinical isolate	Riley et al., 1983
933cu	~90 kb plasmid-cured derivative of 933	Karch et al., 1987
86-24JBK	O157:H7 serotype wild-type, <i>eae</i> positive	obtained from Dr. J. Kaper
86-24m	<i>escN</i> mutant of 86-24JBK	obtained from Dr. J. Kaper

Table 2. continued

Strains	Relevant characteristics	References
Mini-Tn5<i>phoACm</i>^r mutants isogenic to B2F1		
30.10.a	~90 kb plasmid::mini-Tn5 <i>phoACm</i> ^r this study	
34.3	<i>hns</i> ::mini-Tn5 <i>phoACm</i> ^r 1	this study
34.7	<i>hns</i> ::mini-Tn5 <i>phoACm</i> ^r 2	this study
S11GC35	<i>hns</i> ::mini-Tn5 <i>phoACm</i> ^r 3	this study
S11G3A2	<i>hns</i> ::mini-Tn5 <i>phoACm</i> ^r	this study
2B3	~90 kb plasmid::mini-Tn5 <i>phoACm</i> ^r	obtained from Dr. Melton-Celsa

Table 2 continued

Strains	Relevant characteristics	References
Other <i>E. coli</i> strains used		
C600	<i>rfbD</i> mutation, rough laboratory strain	Silhavy et al., 1984
DH10B	<i>lacZ</i> and <i>recA</i> mutant, rough laboratory strain	Grant et al., 1990 Raleigh et al, 1988
DH10B(p2B3)	carries the ~90 kb plasmid that contains mini-Tn5 <i>phoACm</i> ^r insertion from strain 2B3	this study
DH5 α	<i>lacZ</i> and <i>recA</i> mutant rough laboratory strain	Silhavy et al., 1984
HS	serotype 09:H4 non-pathogenic normal flora isolate	Levine et al., 1978
SH210	Δ <i>phoA8</i> , <i>creC51</i> mutant derivative of <i>E. coli</i> K-12	Schweizer et al., 1983
S17-1(λ pir)	RP42-Tc::Mu-Km::Tn7, lysogenized with λ pir, derivative of <i>E. coli</i> K-12	Simon et al., 1983
WAF100 (pSF4000)	<i>recA</i> and <i>mcrB</i> mutant; derivative of <i>E. coli</i> strain HB101	Welch et al., 1983

Table 3. Plasmids used in this study

Plasmid	Description	Reference
pCVD453	derivative of pSPORT ; contains probe A, a fragment of the locus of enterocyte effacement that encodes <i>ler</i> and <i>orf2</i>	McDaniel et al., 1995
pCVD461	derivative of pBluescript KS (-) contains probe B; a fragment of the locus of enterocyte effacement that encodes <i>escJ</i> , <i>sep2</i> , <i>escV</i>	McDaniel et al., 1995
pCVD443	derivative of pUC19; contains probe C, a fragment of the locus of enterocyte effacement that encodes <i>eae</i>	McDaniel et al., 1995
pCVD460	derivative of pBluescript KS (-); of the locus of enterocyte effacement that encodes <i>espD</i> , <i>espB</i> , <i>escF</i>	McDaniel et al., 1995
pTHK113	derivative of pBR322 that contains the <i>E. coli</i> K-12 <i>hns</i> ; encodes ampicillin resistance	Kawula et al., 1991
pTHK116-7	derivative of pACYC184 that contains <i>hns</i> with a promoter mutation that results in lower levels of H-NS; encodes chloramphenicol resistance	Kawula et al., 1991
pCVD419	derivative of pBR325 that contains probe CVD419 that consists of the 3.4 kb <i>HindIII</i> fragment from p0157 carried by STEC strain 933	Levine et al., 1987
p2B3	~90 kb plasmid::mini-Tn5 <i>phoACm</i> ^r	this study

Table 3. Continued

Plasmid	Description	Reference
pSF4000	derivative of plasmid pACYC184, that contains α - <i>hlyC,A,B,D</i> ; and encodes chloramphenicol resistance	Welch et al., 1983
pCM	plasmid pUC7 that encodes chloramphenicol resistance	obtained from Dr. C. Schmitt
pUT/ mini-Tn5 <i>phoAKm</i> ^r	derivative of suicide vector pGP704; a conjugative plasmid that requires the π protein for replication; carries mini-Tn5 <i>phoAKm</i> ^r , and encodes ampicillin resistance	De Lorenzo et al., 1990
pAM400	derivative of pUT/mini-Tn5 <i>phoAKm</i> ^r in which <i>Km</i> ^r is replaced by <i>Cm</i> ^r	obtained from Dr. A. Melton-Celsa
pBR322	moderate copy number cloning vector that encodes tetracycline and ampicillin resistance	Bolivar et al., 1977
pCR2.1	carries deoxythymidine (T) residues for cloning of Taq-generated PCR products; encodes ampicillin and kanamycin resistance	Clark, 1988 Mead et al., 1991
pAOB100	pCR2.1 cloning vector that contains 1.9 kb PCR DNA amplified from B2F1 that encodes wild-type <i>hns</i> with a portion of the <i>galU</i> and <i>tdk</i> genes that flank <i>hns</i>	this study
pMAK705	derivative of suicide vector pSC101 that has a temperature-sensitive origin of replication, a multiple cloning site that contains <i>lacZ</i> , and encodes kanamycin resistance	Hamilton et al., 1989

Table 3. Continued

Plasmid	Description	Reference
pSTAMP	derivative of suicide vector pMAK705 in which the Km ^r has been replaced with Cm ^r	obtained from Dr. A. Melton-Celsa
pAOB347	derivative of pBR322 that contains the 6 kb <i>Hind</i> III DNA fragment from mutant 34.7, also contains the 3' end of <i>hns::mini-Tn5phoACm^r</i> , and a portion of flanking <i>tdk</i> sequences	this study
pCR1211	pCR2.1 cloning vector that contains 4.3 kb PCR DNA product amplified from mutant 34.7; encodes <i>hns::mini-Tn5phoACm^r</i> , and flanking portions of <i>tdk</i> and <i>galU</i>	this study
pMS1211	derivative of suicide pSTAMP; contains the <i>Bam</i> HI fragment from plasmid pCR1211; encodes <i>hns::mini-Tn5phoACm^r</i> , and a portion of the flanking <i>galU</i> and <i>tdk</i>	this study
pCR358	derivative of pCR2.1 cloning vector; contains 358 bp PCR DNA amplified from B2F1; carries a promoterless internal fragment of <i>hns</i>	this study
pMS358	derivative of suicide vector pSTAMP; contains the <i>Eco</i> RI fragment from plasmid pCR358; carries 358 bp truncated <i>hns</i> gene without the promoter	this study

Qualitative adherence assay. Adherence of *E. coli* to T84 cells, a human colonic epithelial cell line, was assessed by a modification of the method described by Cravioto et al., 1979. Confluent monolayers of T84 cells were grown on tissue-culture treated Permanox chamber slides (Nunc, Naperville, IL). The resultant T84 cell monolayers were not polarized and were used for the initial screen of mini-Tn5*phoA*Cm^r adherence mutants.

T84 cells were polarized by growth for 7-10 days on collagen-coated membranes (Costar, Cambridge, MA). The trans-electrical resistance potential generated by polarized T84 cells was measured with the Millicell-ERS system (Milipore, New Hampshire), and cultures were considered to be polarized when the electrical resistance was $\geq 500 \Omega$. Polarized T84 cells were used to confirm the adherence phenotype of the mutant STEC strains tested as compared with the wild-type. Monolayers of non-polarized or polarized T84 cells were washed three times with Dulbecco's PBS supplemented with Ca²⁺/Mg²⁺ and overlaid with 0.45 ml of adherence assay medium (DMEM/F12 contained 0.4% sodium biocarbonate and 1% mannose). Bacterial cultures were grown overnight under static conditions at 37°C, washed once at 5000 rpm for 10 minutes, the spent LB broth removed, and the bacteria resuspended in the same volume of adherence medium. Cultures were used when they reached an OD₆₀₀ of , and 20 µl of each sample was added to the confluent pre-washed monolayers. Each inoculum was equivalent to $\sim 1 \times 10^7$ colony forming units (CFUs) of bacteria (subsequently confirmed by plate count). The approximate multiplicity of infection (MOI) was 100 bacteria :1 tissue culture cell. The inoculated monolayers were incubated for 3 hours to 5 hours at 37°C in a 5% CO₂ atmosphere. A 3 hour adherence assay was used to screen for mini-Tn5*phoA*Cm^r adherence mutants. Mutants that exhibited reduced capacity to bind T84 cells in this assay

were re-tested in a longer 5 hour assay. The monolayers were washed 8 times after the 3-5 hour incubation to remove non-adherent bacteria. Each wash was removed by aspiration with a Pasteur pipette attached to a vacuum flask. Assays were done in duplicate and repeated at least three times. Slides and transwell membranes were fixed and stained with Leukostat available from (Fisher Scientific, New Hampshire). Leukostat is a modified Wright's stain. With this stain, bacteria are dark blue in contrast to the pink color of T84 cells.

Fluorescence actin staining assay (FAS). *E. coli* 0157:H7 strains and other LEE- positive STEC cause actin polymerization at the site of bacterial attachment as demonstrated by a positive FAS test (Knutton et al., 1989). STEC 091:H21 strain B2F1 does not adhere well to HEP-2 cells, (McKee et al, 1995); therefore, the FAS phenotype on HEP-2 cells of B2F1 can not be determined. To assess the FAS phenotype of B2F1 adherent to T84 cells after a 5 hour adherence assay, the FAS (Sigma St. Louis, MO) staining procedure of Knutton et al., 1989 was used.

Quantitative adherence assay. Bacteria were allowed to adhere to polarized or non-polarized T84 cells for 3 hours. Specifically, non-polarized T84 cells were used for initial quantitative analysis of adherence mutants, and results were confirmed with polarized cultures of T84 cells. Monolayers were washed extensively, then treated with 0.25% trypsin (0.45ml) in PBS (without Ca^{2+} or Mg^{2+}) for 10 minutes at 37°C. The trypsinized, infected monolayers were aspirated from the wells and the suspensions were mixed vigorously with a 1ml eppendorf pipette to disrupt clumps and to dislodge bacteria from the T84 cells. These bacteria-containing lysates were serially diluted in PBS then plated onto LB agar (with or without antibiotics as appropriate) to obtain colony forming units (CFUs). Dilutions that gave

CFUs between 30 and 300 were used to calculate the number of adherent bacteria per ml for each sample tested. Total number of CFU per ml (CFU/ml) = (CFU on agar plate) x (dilution factor) x 10 (factor of 0.1 ml of mixture plated onto agar) x 2 (factor for 0.45 ml trypsin solution added).

Isolation of plasmids. Small-scale preparations of plasmid DNA were obtained by the alkaline lysis procedure (Birnboim et al., 1979). Plasmid DNA samples that were used for cloning procedures and in automated sequencing reactions were purified by the Qiagen miniprep technique (Qiagen Inc., Chatsworth, CA). The 90 kb plasmid from strain B2F1 was isolated by the method of Kado and Liu (1981) and electroporated into DH5 α and DH10B or used for *in situ* hybridization experiments. The Kado and Liu procedure incorporates alkaline conditions at pH 12 followed by a 60 minute incubation period at 65° C to denature chromosomal DNA. Large plasmids isolated by this procedure are free of chromosomal DNA, highly stable, and suitable for transformation, cloning, and *in situ* DNA-DNA hybridization experiments. Following treatment of B2F1 by the Kado and Liu method, plasmids were either transformed into host strains that had been treated with CaCl₂ (Mandel and Higa, 1970) or moved into strains by electroporation (Dower et al., 1998) at 1.25kV, 25 μ F, 1000 Ω with a Bio-Rad Pulse Controller (Bio-Rad, Hercules, CA).

Isolation of total DNA. Bacterial DNA was isolated by the procedure of Ausubel et al., 1990. A saturated 5 ml bacterial culture was centrifuged, and the pellet was resuspended in 567 μ l of Tris-EDTA buffer after which 30 μ l of 10% SDS and proteinase K was added. The mixture was incubated for one hour at 37° C. Addition of CTAB (hexadecyltrimethyl ammonium bromide), 100 μ l of 5 M NaCl, and a 10 minute incubation at 65° C removed cell

debris and denatured proteins from solution. The mixture was extracted once with chloroform-isoamyl alcohol, twice with phenol:chloroform, and then subjected to centrifugation. The DNA in this suspension was precipitated by the addition of isopropanol, washed twice with 70% ETOH, and then resuspended in water.

Recombinant DNA methods. Restricted DNA fragments were separated by agarose electrophoresis. DNA fragments were purified from agarose gel slices as follows. DNA fragments that were greater than 500 bp were eluted from the gel matrix with the GeneClean spin system (Bio 10, Vista, CA), and fragments less than 500bp were eluted with the Qiaex II gel extraction kit (Qiagen, inc., Chatsworth, CA). The purified DNA fragments were ligated into the appropriate vector with T4 DNA ligase (Invitrogen, Carlsbad, CA). Alternatively, specific primers were used to generate PCR products from the bacterial strain of interest with the GeneAmp kit manufactured by Applied Biosystems, Inc., Foster City, CA. PCR products were separated by agarose gel electrophoresis and then purified from the agarose as described above. These isolated PCR fragments were then ligated into the appropriate vector. The fragment identity was checked by restriction digest analysis and/or by sequence analysis.

Southern blot analysis. Total DNA was digested with the appropriate restriction enzyme. The restricted fragments were separated by agarose gel electrophoresis (0.6 or 0.7 %) and subjected to *in situ* gel hybridization as described by Kidd et al.,1983. Gels were run overnight at 25 volts, stained with ethidium bromide, and photographed after electrophoresis. The DNA in each gel was depurinated with 250 mM HCl, denatured with 0.5 M NaOH, and neutralized to pH 7.5 with Tris buffer before transfer to a nytran plus membrane (Schleicher

& Schuell, Keene, NH). Transfer of the DNA was achieved with the rapid downward transfer system (Turboblotter, sold by Schleicher & Schuell) that is based on the methods of Southern, 1975 and Chomczynski, 1992. The DNA, after transfer, was fixed to the blot by cross-linking with ultra violet (UV) light (Church and Gilbert, 1984). The blot was blocked at least 4 hours before addition of labeled probe (see below). Routinely, the probe was hybridized to target DNA for 24 hours at 42°C. The blot was washed twice at 42°C with buffer that contained 0.0075 M sodium citrate plus 0.075 M NaCl, pH 7.0 (0.5x SSC) and 6 M urea, followed by a final wash with 20x SSC at room temperature. The ECLTM detection reagents (Amersham Life Science) were added, and the blot was subjected to autoradiography.

The probe was prepared from agarose gel purified restriction DNA fragments labeled with the ECLTM nucleic acid kit by the procedures of Renz and Kurz, 1984. The system uses direct coupling of the DNA probe to horseradish peroxidase. The labeled probe hybridizes with target DNA, and upon addition of detection reagents that contain hydrogen peroxide and luminol, a blue light is emitted that is captured on blue-light sensitive film. The positive hybridization result appears as a dark band after the film is developed.

Colony blot analysis. Overnight cultures were diluted and plated onto LB media (with or without supplements) to yield a low density of colonies per plate. Plates were chilled to 4°C for one hour prior to conducting colony lifts. Positively charged nylon membranes (Dupont NEN Research Products, Boston, MA) were used for colony hybridization experiments. Membranes were allowed to sit on the agar plate for 2-3 minutes, and the orientation of the disc position was marked. Membranes were then placed colony side up for 2-5 minutes onto a pool of 0.5 N NaOH which lysed the bacteria. The membranes were rinsed

three times in 500 ml of 5x SSC buffer to remove cellular debris. Washed membranes were dried, and the DNA was cross-linked by UV light. Hybridization procedures were performed and probe was prepared as described above under Southern blot analysis.

DNA sequencing. Nucleotide sequence analysis was done by the dideoxy chain termination method (Sanger et al., 1977). DNA inserts from recombinant plasmids pAOB347 and pCR1211 were sequenced with the the Prism Ready Reaction Cycle Sequencing kit (Applied Biosystems, Inc.) according to the manufacturer's protocol. The products of the automated sequence reaction were separated and analyzed on an Applied Biosystems Model 373 Automated DNA sequencer (ABI, Inc.)

Generalized transduction of B2F1 with P1::Tn9 bacteriophage.

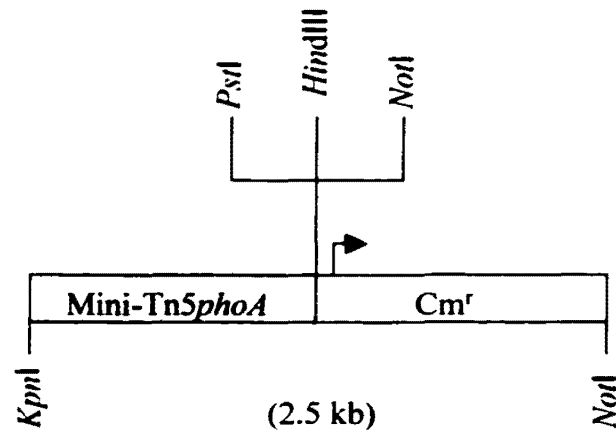
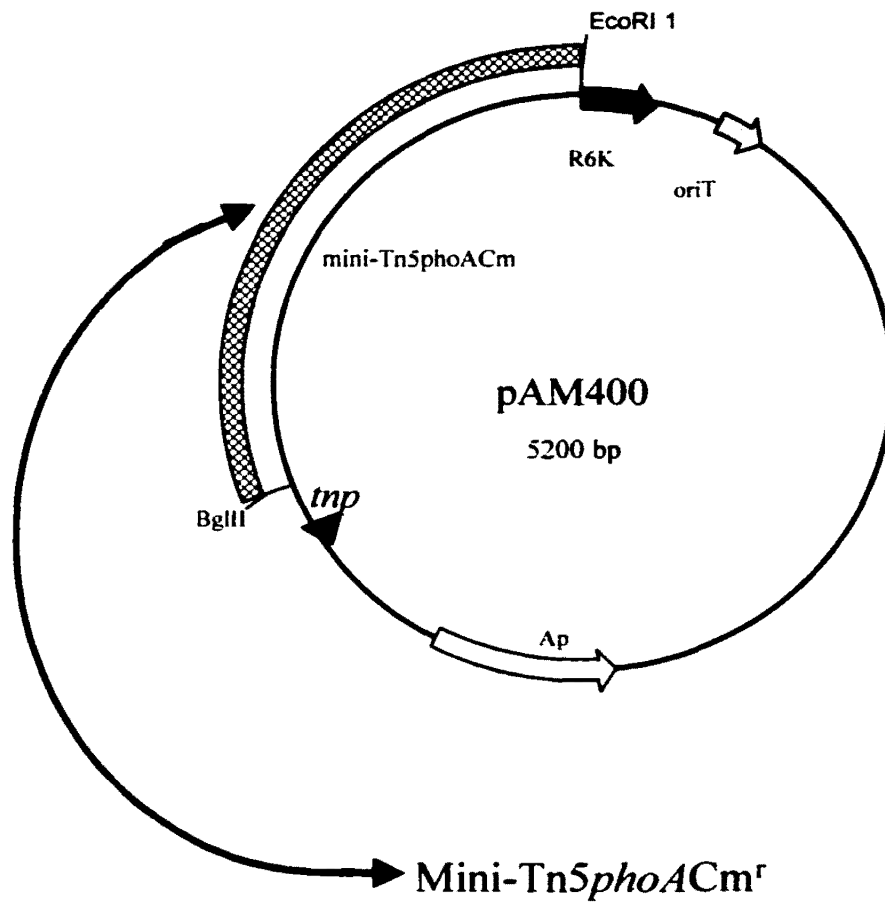
P1::Tn9 bacteriophage (McCormick et al., 1989) encodes a temperature-sensitive repressor that at 42°C becomes inactive and induces the lysogenic phage to become lytic. The Tn9 insertion within the repressor of this P1 phage carries a chloramphenicol resistance gene that serves as a selectable marker. Previously, this phage was used to transduce a *leuX*⁻ mutation into B2F1 (Dr. Paul Cohen 1996 personal communication). *E. coli* strain SH210 (Schweizer & Boos, 1983) is an alkaline phosphatase (*phoA*) null mutant. In SH210, this mutation is linked to Tn10. The Tn10 insertion confers tetracycline resistance to SH210. P1 transduction of the *phoA* mutation that is linked to Tn10 from SH210 into STEC strain B2F1 was attempted to obtain a negative PhoA background for mini-Tn5*phoA*Cm^r mutagenesis. High-titer lysates, of approximately 1 x 10¹¹ plaque forming units/ml (PFU) P1::Tn9 were prepared on *E. coli* strain SH210. B2F1(the recipient) was grown to log-phase, and approximately 10⁸ CFU/ml cells were harvested by centrifugation. LB broth with 250 mM

CaCl and the P1::Tn9_(SH210) lysate was added to the pellet of B2F1, so that the multiplicity of infection was 100 PFU :1 bacterium. Adsorption of the phage to B2F1 was done for one hour at 30°C without shaking. The culture was then concentrated by centrifugation at 7000 rpm, and the pellet was then resuspended in 1ml of fresh broth with tetracycline and further incubated at 37°C for 24 hours. A 100 µl sample of the culture was applied to agar with 0.5 mM sodium citrate and 12.5 µg/ml of tetracycline and the surviving bacteria were permitted to grow overnight at 42°C. The following day, the cultures were replica plated onto media with tetracycline or chloramphenicol. Colonies of B2F1 that were resistant to tetracycline but sensitive to chloramphenicol and white in the presence of XP substrate were presumed to have been successfully transduced with P1::Tn9 and to have acquired the Tn10 insertion along with the alkaline phosphatase mutation from strain SH210.

Description of the mini-Tn5*phoA*Cm^r transposon and the delivery system utilized for mutagenesis. The mini-Tn5*phoA* transposon was developed by De Lorenzo et al., 1990 and encodes a kanamycin (Km^r) resistance gene. Dr. Melton-Celsa, replaced Km^r with the chloramphenicol resistance gene (Cm^r) because spontaneous B2F1 kanamycin resistant mutants of B2F1 arise with a high frequency (unpublished observation).

The delivery system, plasmid pAM400, used for our modified mini-Tn5*phoA*Cm^r is illustrated in Figure 4. Plasmid pAM400 is a derivative of pUT/mini-Tn5*phoA*Km^r and is a derivative of the suicide vector pGP704 that was constructed by Miller and Mekalanos in 1988. The origin of replication for all of these related plasmids is the π protein-dependent origin of the plasmid R6K (Kolter et al., 1978). The π protein-dependent origin can only be maintained in bacteria that produce the π protein. Plasmid pAM400 also carries the origin of

Figure 4. Schematic of plasmid pAM400 that carries the mini-Tn5*phoACm*^r mobile element. The hatched area of the plasmid represents the mini-Tn5*phoACm*^r. Plasmid pAM400 encodes ampicillin resistance (Ap), has a π protein-dependent origin of replication (R6K), carries the origin of transfer (*oriT*) for mobilization of pAM400 into compatible recipients and encodes transposase (*mp*) employed for transposition of the mini-Tn5*phoACm*^r element. Mini-Tn5*phoACm*^r, drawn below the plasmid, encodes the *E.coli* alkaline phosphatase gene that does not contain a signal sequence or ribosomal binding site and can generate hybrid proteins that exhibit PhoA activity when fused in-frame with a gene that encodes exported proteins. The transposon also confers chloramphenicol resistance (Cm^r) and the small black arrow indicates the direction of transcription of the Cm^r gene.



transfer, *oriT*, of the plasmid RP4. The presence of *oriT* allows the efficient conjugal transfer to gram-negative recipient strains from donor strains that express RP4-conjugative functions (Miller and Mekalanos, 1988). In addition, these plasmids carry *tnp*, a mutant gene of Tn5 IS50R that encodes the transposase needed for transposition of the mini-Tn5*phoACm'* element. This transposase is encoded outside of the mobile unit of the mini-Tn5*phoACm'*, does not transfer to the recipient strain during transposition, and is lost when the suicide plasmid is cured from the recipient strain. The pAM400 plasmid also encodes an ampicillin resistance gene that is independent of the mobile mini-Tn5*phoACm'* unit.

The host strain carrying the suicide plasmid that encodes the mini-Tn5*phoACm'* is S17-1(λ *pir*). This strain, a descendent of *E. coli* strain 294, was constructed by Simon and colleagues (Simon et al., 1983). Strain S17-1(λ *pir*) is DNA restriction system minus and is lysogenized with a λ bacteriophage that contains the *pir* gene from plasmid R6K. Therefore, S17-1(λ *pir*) encodes π protein that allows maintenance of R6K-based plasmids in this host. Moreover, transfer genes encoded by the RP4-2 plasmid integrated into the chromosome of strain) supply the trans-acting factors that allow S17-1(λ *pir*) to mobilize plasmid pAM400 and related plasmids into a recipient strain with high frequency.

Procedure for transfer of mini-Tn5*phoACm'* by conjugal mating.

Mutagenesis of nalidixic acid-resistant B2F1 with mini-Tn5*phoACm'* was carried out as follows. The recipient B2F1 and the *E. coli* donor S17-1(λ *pir*)/pAM400 were grown overnight at 37 °C in Terrific broth (Maniatis, 1989) that contained either nalidixic acid (for B2F1) or chloramphenicol [for S17-1(λ *pir*)/pAM400]. Each culture was then diluted 1:50 in fresh antibiotic-free Terrific broth. The recipient B2F1 culture was grown for one hour with

aeration at 37°C and then the culture was shifted to 30°C with agitation. The S17-1(λ *pir*)/pAM400 culture was also grown at 37°C for one hour with shaking and then the culture was shifted to static conditions at 37°C. Growth of both cultures was permitted to continue until each had reached mid log-phase. The OD₆₀₀ was 0.5 for both the donor and the recipient before mating. B2F1 (50 μ l) was applied onto pre-warmed LB agar and dried at 37°C before 50 μ l of S17(λ *pir*)/pAM400 was placed on top of the dried spot that contained the recipient culture. Mating was allowed to proceed for one hour or 24 hours. The mating mixture was resuspended in 1 ml of LB broth that contained 10 mM MgSO₄. This mixture (100 μ l) was then spread onto plates that contained both chloramphenicol and nalidixic acid to select for recipient cells (B2F1) that had acquired the transposon marker (chloramphenicol). This selection medium was also supplemented with 80 μ g/ml of XP substrate. Colonies that were blue after 15-18 hours were presumed to have undergone an in-frame insertion of the mini-Tn5*phoACm'* within a gene that encoded an outer membrane, periplasmic, or secreted protein. These colonies were patched onto plates supplemented with chloramphenicol or ampicillin and scored the next day for sensitivity to ampicillin. Colonies that were ampicillin sensitive (reflecting a loss of pAM400) were picked, and the adherence phenotype of the organism grown from those colonies was assessed by the standard 3 hour adherence assay.

Assay for extracellular hemolytic activity. A modification of the method of Welch et al., 1983 was used to quantitate hemolytic activity in bacterial culture supernatants. Bacterial strains were grown overnight, diluted 1:50 into fresh media, and grown with agitation to log phase (OD₆₀₀ of 1.0). These cultures were centrifuged at 5000 rpm for 15 minutes to pellet bacteria. The supernatants were passed through a low protein-binding 0.45 μ m Milipore

filter to remove residual bacteria. The supernatants were kept on ice prior to incubation with sheep red blood cells (SRBC) that had been washed three times with 0.1M PBS by low speed centrifugation at 4°C. The washed SRBCs were maintained in a buffer that consisted of 0.1 M PBS with 10 mM CaCl₂ and 0.1% BSA and used at a final v/v concentration of 2%. Each 2-fold serial dilution of bacterial filtrate (500 µl) was added slowly to the side of a 1.5 ml eppendorff tube that contained 500 µl of the washed 2% SRBC suspension. After 2 hours of incubation at 37°C, SRBCs were pelleted by a quick 30 second spin in a microcentrifuge set at maximum speed. The supernatant was removed and its optical density reading at 540 nm was taken. A buffer control that consisted of the suspension buffer plus 2% SRBC at a 1:1 ratio was included in the assay as the negative control for hemolysis. The positive control (maximum lysis) contained 500 µl of water and 500 µl of 2% SRBC. In addition *E.coli* K-12 strain DH5α does not produce hemolysin and was included as a bacterial culture negative control.

Detection of hemolysis on blood agar plates. Hemolysis of SRBC by wild-type STEC 0157:H7 can not be detected on blood agar plates prepared commercially (Beutin et al., 1989; Melton-Celsa unpublished observation). However small turbid zones around strains of 0157:H7 and B2F1 are evident when these bacteria are grown on tryptose blood agar base (Difco Laboratories) supplemented with 10 mM CaCl₂ and 5% defibrinated sheep blood (Cocalico). The sheep red blood cells used in these plates are pre-washed three times in PBS at pH 7.2 (Beutin et al 1989). These washed SRBC plates were used to compare the hemolytic phenotype of wild-type STEC strains with the mini-Tn5*phoAC*^{cm^r} mutants. Otherwise, 5% SRBC supplemented agar plates (Difco) were used to assess the hemolytic phenotype of the

mutants because various mutants, which will be described in detail, produced zones of clearing around the colony even when unwashed blood was incorporated into the media.

Preparation of bacterial lysates for hemolysin detection by Western

blot analysis. Stationery phase cultures of *E. coli* were diluted to an equivalent optical density. Samples (2 ml) of each culture were centrifuged at 5000 rpm. The resulting supernatants were separated from the bacterial pellet and placed on ice. Bacterial pellets were suspended in 2 ml of 0.1 M PBS at 4°C. The pellet and whole culture (2 ml) samples were lysed by sonic disruption. Proteins from the three types of samples (pellet, supernatant, and whole culture) were then precipitated with trichloroacetic acid (10% TCA final concentration per sample v:v) by rocking samples overnight at 4°C. The precipitated protein was recovered from the samples by centrifugation at 10,000 x g for 10 minutes. The protein precipitates were washed with cold acetone, the pellet dried slightly, and resuspended in denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were pre-heated to 100°C before they were loaded onto SDS-PAGE gels.

SDS-PAGE of these TCA precipitated samples was done with the Mini-Protean II slab cell according to the instruction manual provided by the manufacturer (Bio-Rad, Richmond, CA). Discontinuous slab gels (Laemmli et al., 1970) with a 7.5 % acrylamide resolving gel and 4% stacking gel were used. Gels were either fixed in 12% TCA before they were stained with colloidal G-250 coomassie brilliant blue (Sigma, St. Louis, Mo) or electroblotted onto nitrocellulose (Towbin, 1979) with a trans-blot SD semi-dry apparatus (Bio-Rad). The membranes were blocked with 5% nonfat dried milk (Carnation Co., Los Angeles, CA) in Tris-buffered saline, pH 7.2 with 0.1% Tween-20 (TBS-T). The blots were incubated for 24 hours

at 4°C with rabbit anti- α -hemolysin polyclonal sera. After incubation with the primary antibody and three washes with TBS-T, the blots were overlaid with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody. The immunoblots were washed, and the antigen-antibody complexes were visualized by chemiluminescence with the ECLTM Western blotting detection kit (Amersham).

Vero cell cytotoxicity assay. The Vero cell cytotoxic assay is routinely used in this laboratory to assess the cytotoxic activity of Shiga toxin (Stx) produced by isolates of STEC. Filtrates of bacterial culture supernatants or sonic lysates were tested for Vero cell cytotoxicity by the method of Gentry and Dalrymple (1980) as modified by Schmitt et al., 1991. The cytotoxic titer per milliliter of culture is expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a given well (CD_{50}/ml).

Evaluation of motility of mutants in semi-solid agar. Tryptone swarm plates that contained 1% Bacto-Tryptone (Difco), 0.5% NaCl, and 0.3% Bacto-Agar (Difco) were prepared with or without chloramphenicol or ampicillin added. Strains to be tested were grown overnight at 37°C without agitation. Static overnight cultures contained approximately 10^9 CFU/ml. Swarm plates were inoculated with ~5 μ l stab of the overnight culture (~ 10^6 organisms). Plates were incubated at 30°C for 15-18 hours. Swarm diameters (measured in millimeters) of *hns* mutants were compared to swarm diameters of wild-type B2F1 and the plasmid-cured derivative S11. A swarm rate of 1 indicated identical swarm diameter sizes between the two strains for which the rates were compared. Experiments were done with *hns* mutants and *hns* wild-type strain in triplicate on three separate occasions.

Methods used to compare membrane barrier function of wild-type

B2F1 to mini-Tn*SphoA*Cm^r mutants of B2F1. Growth of wild-type and mutant strains in the presence of crystal violet was done to test for normal outer-membrane barrier function. A 1:100 dilution of overnight cultures of each strain was mixed with cooled soft agar, poured into sterile petri dishes, the agar was allowed to solidify, and the filter that contained the crystal violet was placed in the center of the plate. The plates were incubated at 37°C overnight and observed the next day. Growth of organisms up to the edge of the crystal violet soaked disk indicates that the membrane can exclude molecules that are not normally transported into the cell.

In addition, wild-type and mutant strains were streaked for isolation on LB agar that contained 3% instant non-fat dry milk (source of casein) and incubated overnight at 37°C. After 18 hours of incubation, plates were examined for zones of clearing around individual colonies. A zone of clearance around colonies would indicate that proteases leaked from the cell in an abnormal manner and suggest that outer membrane function is impaired.

Results

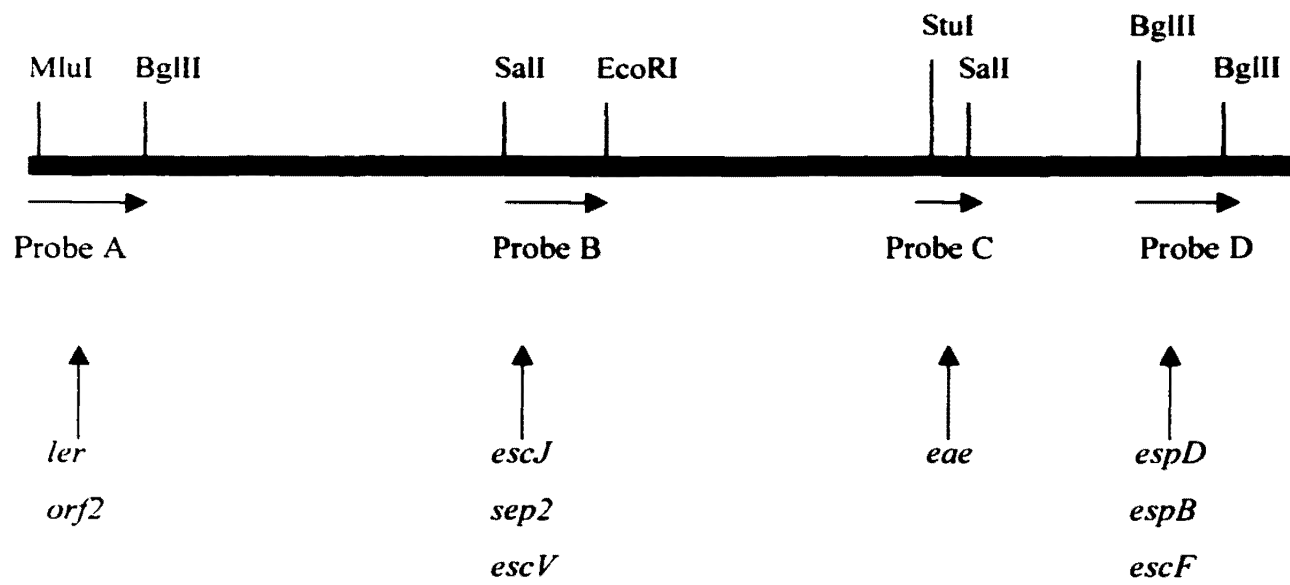
I. Absence of the locus of enterocyte effacement in STEC prototype strain B2F1.

The main goal of this investigation was to identify factor(s) important for the adherence to human colonic epithelial cells of the intimin-negative, STEC strain B2F1. Although previous studies revealed that B2F1 was *eae* negative by Southern blot hybridization analysis (McKee, 1995), we did not know at the onset of these studies whether the strain encoded any or all of the rest of the genes within the 35 kb locus of enterocyte effacement (LEE) present in EPEC E2348/69. Therefore, strains of DH5 α that contained high-copy number plasmids that carried LEE-specific probes were obtained from Tim McDaniel at the Center for Vaccine Development, Department of Medicine at the University of Maryland, Baltimore. The probes consisted of four DNA restriction fragments that span the 35 kb LEE and encode some of the genes demonstrated by others (McDaniel et al., 1995; Mellies et al., 1999) to be essential for the attaching and effacement (A/E) phenotype of EPEC E2348/69 and EHEC 0157:H7 (Kaper et al., 1998). The regions of the LEE that constituted probes A-D are shown in Figure 5. Previously, McDaniel and colleagues found that “DNA sequences encoded by the LEE probes were either all present or absent in a given strain and that the probes were 100% sensitive and 98.7% specific for strains with the A/E phenotype” (McDaniel et al., 1995). To determine whether strain B2F1 carried any essential LEE genes,

Figure 5. Restriction fragments derived from the EPEC 35 kb Locus of Enterocyte Effacement (LEE) that constituted the DNA probes used for colony blot hybridization of wild-type B2F1. Probe A is comprised of *ler*, the LEE encoded regulator and *orf2*, unknown function; this probe contains regions 676-1494 bp and 1622 -2818 bp, respectively of the LEE. Three genes important for function of the type III secretion system are represented by probe B, *escJ* (3331-13903), *escV* (14364-14660 bp), and *sep2* (14364-14660 bp). The structural gene of intimin, *eae* (24849-27668 bp) comprises probe C. The genes included in probe D are *espD* (30952-32094 bp), *espB* (32115-33080 bp), and *escF* (33531-33752 bp). These three genes are important in the signal transduction events involved in adherence of intimin-positive bacteria.

LEE pathogenicity island

35624 bp



each of the 4 probes was hybridized to DNA from isolated colonies of strain B2F1 by colony blot analysis. Enterohemorrhagic *E. coli* 0157:H7 and enteropathogenic *E. coli* 0127:H6 strains that encode the LEE locus were used as the positive controls and the laboratory strains DH5 α and S17-1(λ *pir*) served as negative controls. The positive control strains hybridized with all four probes while the negative control strains, DH5 α and S17-1(λ *pir*) did not nor did B2F1 (Figures 6 and 7). Thus, B2F1 does not encode any of the 4 regions of the LEE locus detected by probes A-D.

II. *In vitro* model of adherence of STEC to human colonic epithelial cells.

Human colonic epithelial cell line used to optimize the *in vitro* adherence assay. The HEp-2 epithelial cell line that was derived from human larynx has been used extensively to study the localized adherence (LA) of intimin-producing EPEC and EHEC. The localized adherence pattern involves the accumulation of filamentous actin at the site of bacterial attachment, and this actin accumulation is detected by fluorescein-conjugated phalloidin staining of actin in HEp-2 cells (Cravioto et al., 1979; Knutton et al., 1989; Jerse, et al., 1990). EHEC require intimin to adhere to HEp-2 cells *in vitro* and to produce the FAS phenotype. (McKee et al., 1995). However, STEC group 1 and 2 isolates do not encode the gene for intimin. Indeed McKee et al. (1995) showed that strain B2F1 does not adhere well to HEp-2 cells. However, B2F1 was originally isolated from the feces of a patient with HUS, and therefore,

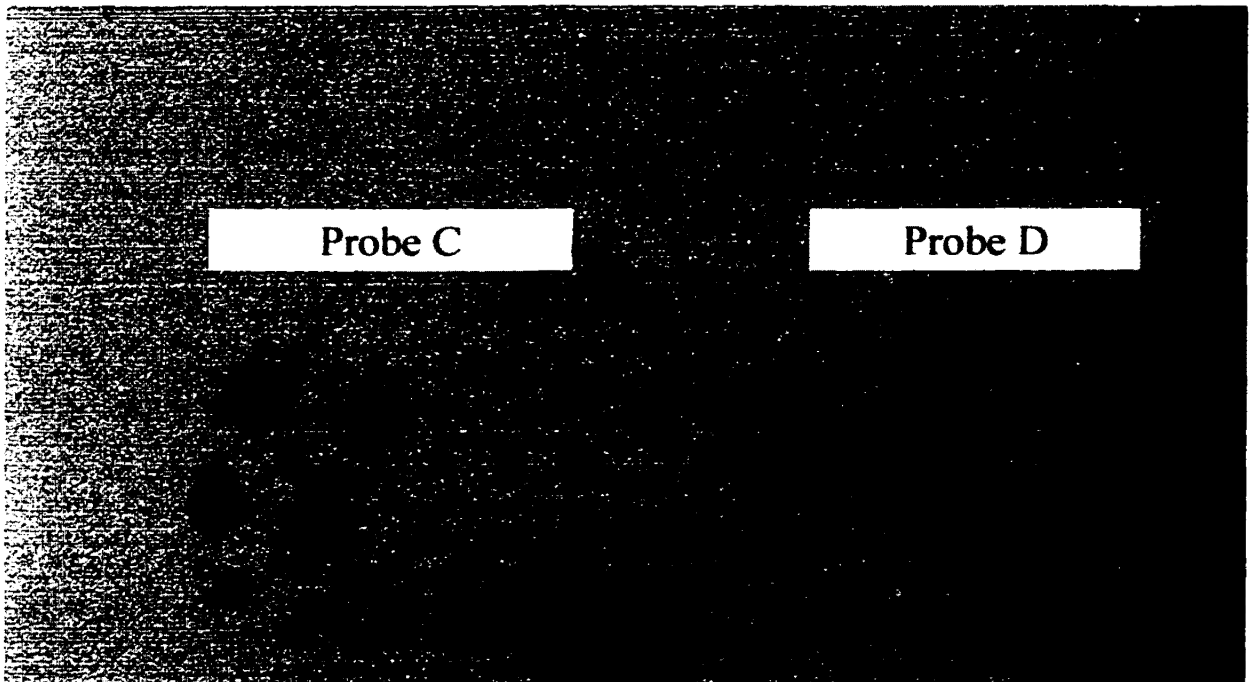
Figure 6. Colony blot hybridization of STEC strain B2F1 with restricted DNA fragments derived from the locus of enterocyte effacement (LEE), probes A and B. Probe specifications are described in detail in Figure 5. Positive control strains known to encode the LEE are EHEC strain 86-24 in position 1-2 and EPEC strain E2348/69 in positions 5-6. Probe A (position 13-14) and probe B (position 15-16) are positive controls for probe reactivity. Negative controls are two laboratory strains of *E. coli*, DH5 α and S17-1(λ pir) (positions 7-10). B2F1 is always in positions 11 and 12. Position 3 and 4 were not used.



Probe A

Probe B

Figure 7. Colony blot hybridization of STEC strain B2F1 with restricted DNA fragments derived from the locus of enterocyte effacement (LEE), probes C and D. Probe specifications are described in detail in Figure 5. Positive control strains known to encode the LEE are EHEC strain 86-24 in position 1-2 and EPEC strain E2348/69 in positions 5-6. Probe C (position 15-16) and probe D (position 15-16) are positive controls for probe reactivity. Negative controls are two laboratory strains of *E. coli*, DH5 α and S17-1(λ pir) (positions 7-10). B2F1 is always in positions 11 and 12. Positions 3 and 4 were not used.



must have persisted in the human gastrointestinal tract for about 8 days (mean time to development of HUS from time of infection). Therefore, we hypothesized that B2F1 has the capacity to adhere to human colonic epithelial cells by an intimin-independent mechanism. We proposed to test our hypothesis by conducting our B2F1 adherence assay on an epithelial cell line of human colonic origin.

The T84 human colonic adenocarcinoma cell line is one of the very few cell lines that resembles *in vitro* the native intestinal cell from which it was derived, the colonic crypt cell (Dharmathaphorn and Madara, 1990). T84 cells can undergo polarization and form tall columnar epithelial monolayers followed by the formation of intercellular occluding junctions (tight junctions) and desmosomes. T84 cells mature both structurally and functionally with the formation of transepithelial monolayer resistance, a barrier to paracellular flow and normal distribution of various proteins to apical and basolateral compartments. Thus, T84 cells when grown *in vitro* may resemble the *in situ* microvilli of the intestine (Madara and Dharmathaphorn, 1985). Furthermore, T84 cells are not sensitive to killing by Shiga toxin because they lack the toxin binding receptor (Philpott et al., 1997). For these reasons, T84 cells were used in this study to establish an *in vitro* model of adherence to human colonic epithelial cells by STEC.

EHEC and STEC strains used to optimize the adherence assay. Table 4 describes the clinical isolates and control *E. coli* strains used to establish the adherence assay. The clinical isolates that were chosen as positive controls included three intimin-encoding isolates. EHEC 0157:H7 strain 933 and EHEC 0157:H7 strain 86-24, and EHEC

Footnotes for Table 4.

^a Strain 933 and 86-24 are both EHEC group 1 serotype 0157:H7 clinical isolates. 933cu is the plasmid-cured derivative of 933 and 86-24 $eae\Delta 10$ is an *eae* deletion mutant of 86-24. H30 is an EHEC group 2 member, serotype 026:H11. Clinical isolate B2F1 belongs to STEC group 1, is intimin negative, encodes two copies of Stx2d (Stx2d1 and 2d2) and serotype 091:H21. HS is a nonpathogenic intestinal flora isolate of *E. coli* of serotype 09:H4 and DH5 α is a rough K-12 laboratory strains of *E. coli*.

^b The presence of the ~90 kb plasmid was determined by hybridization with the 3.4 kb *HindIII* fragment derived from p0157 carried by EHEC 0157:H7, and is designated as probe CVD419 (Levine et al., 1987).

^c The toxin phenotype was determined by neutralization of Vero cell cytotoxicity with anti-Shiga toxin 1(Stx 1) or anti-Stx2 antibodies. Stx 2d1 and Stx 2d2 are variant forms of Shiga toxin 2 that can be detected with antibodies against Stx 2 and these toxins can be activated by human intestinal mucous (Melton-Celsa et al., 1996).

^d HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome; N/A not applicable.

Table 4. Characteristics of bacterial strains used to optimize the in vitro model of adherence to T84 cells by STEC organisms

Strain ^a	(~90) kd Plasmid present ^b	Produces intimin	Stx type ^c produced	Clinical findings ^d
933	+	+	1 and 2	HC
933cu	-	+	1 and 2	N/A
86-24	+	+	2 only	HC
85-24 <i>eae</i> Δ 10	+	-	2 only	N/A
H30	+	+	1 only	HC
B2F1	+	-	2d	HUS
HS	-	-	none	N/A
DH5 α	-	-	none	N/A

O26:H11 strain H30. Strain B2F1 served as the intimin-negative STEC strain. A nonpathogenic *E. coli* K-12, strain DH5 α , was used in most experiments as a strain to define the background level of bacterial adherence in this system. HS, an intestinal flora isolate of *E. coli*, was included in an initial assay (Levine et al., 1978). Mannose blocks type 1 fimbrial binding of K-12 strains to epithelial cells *in vitro* (Ofek et al., 1977) and was included in each adherence assay so that type 1 fimbrial-independent binding of pathogenic *E. coli* to T84 cells could be assessed.

Qualitative analysis of STEC adherence to T84 cells To test the adherence assay a trial run was performed with HEp-2 cells as described previously (McKee et al., 1995) and B2F1 failed to bind to HEp-2 cells as had been shown by Dr. McKee (McKee et al., 1995). Also, as previously demonstrated (McKee et al., 1995), the intimin-positive strain 86-24 formed small microcolonies on HEp-2 cells and was FAS positive (Figure 8). These confirmatory findings substantiated the validity of the adherence assay. We next sought to apply the assay to T84 cells.

Semi-confluent cultures of T84 cells were inoculated with B2F1, H30 or DH5 α and incubated for periods of one, three and five hours. Within three hours, the difference in the amount of adherence of B2F1 compared to DH5 α and H30 was readily apparent by phase contrast light microscopy. Isolate H30 required five hours of incubation (Figure 9) before it attached to T84 cells at levels greater than the DH5 α , but B2F1 adhered well even after 3 hours of incubation. All strains, H30, B2F1 and DH5 α , were seen in high numbers attached to the plastic between cells (data not shown).

Figure 8. Phase contrast (panels A and C) and fluorescent (panels B and D) micrographs of HEp-2 cells after a six hour incubation with B2F1 (panels A and B) and 86-24 (panels C and D). Strain 86-24 attached to and formed microcolonies with the accumulation of filamentous actin (arrow panel C and panel D) on HEp-2 cells. B2F1 did not bind to HEp-2 cells as assessed visually. Fluorescein isothiocyanate (FITC)-conjugated to phalloidin was used to stain polymerized filamentous actin.

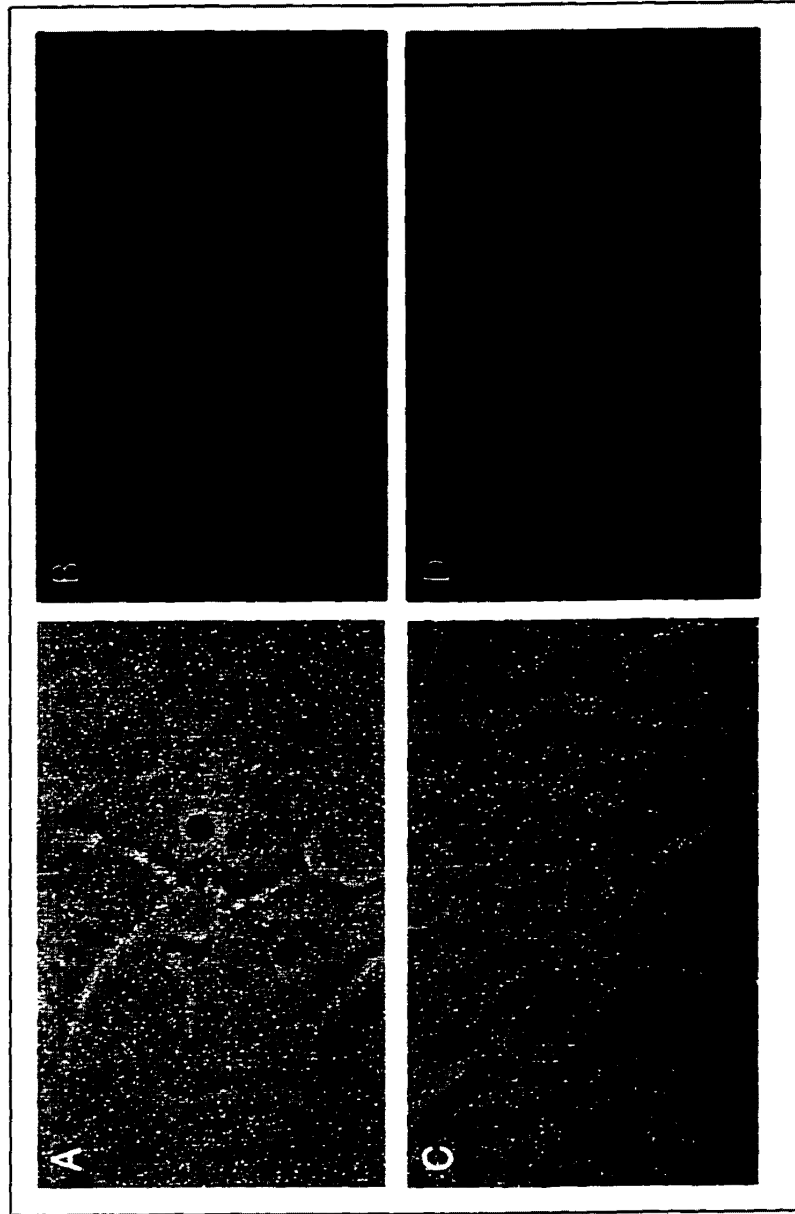
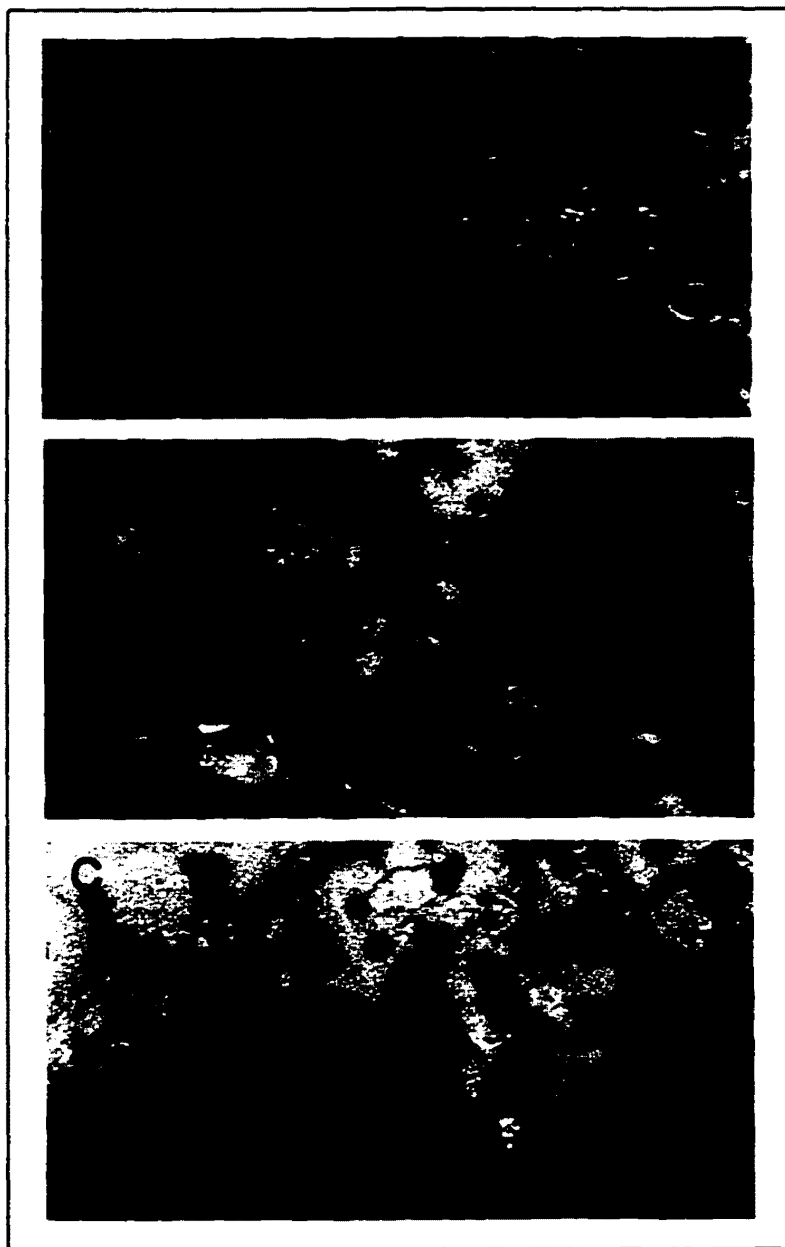


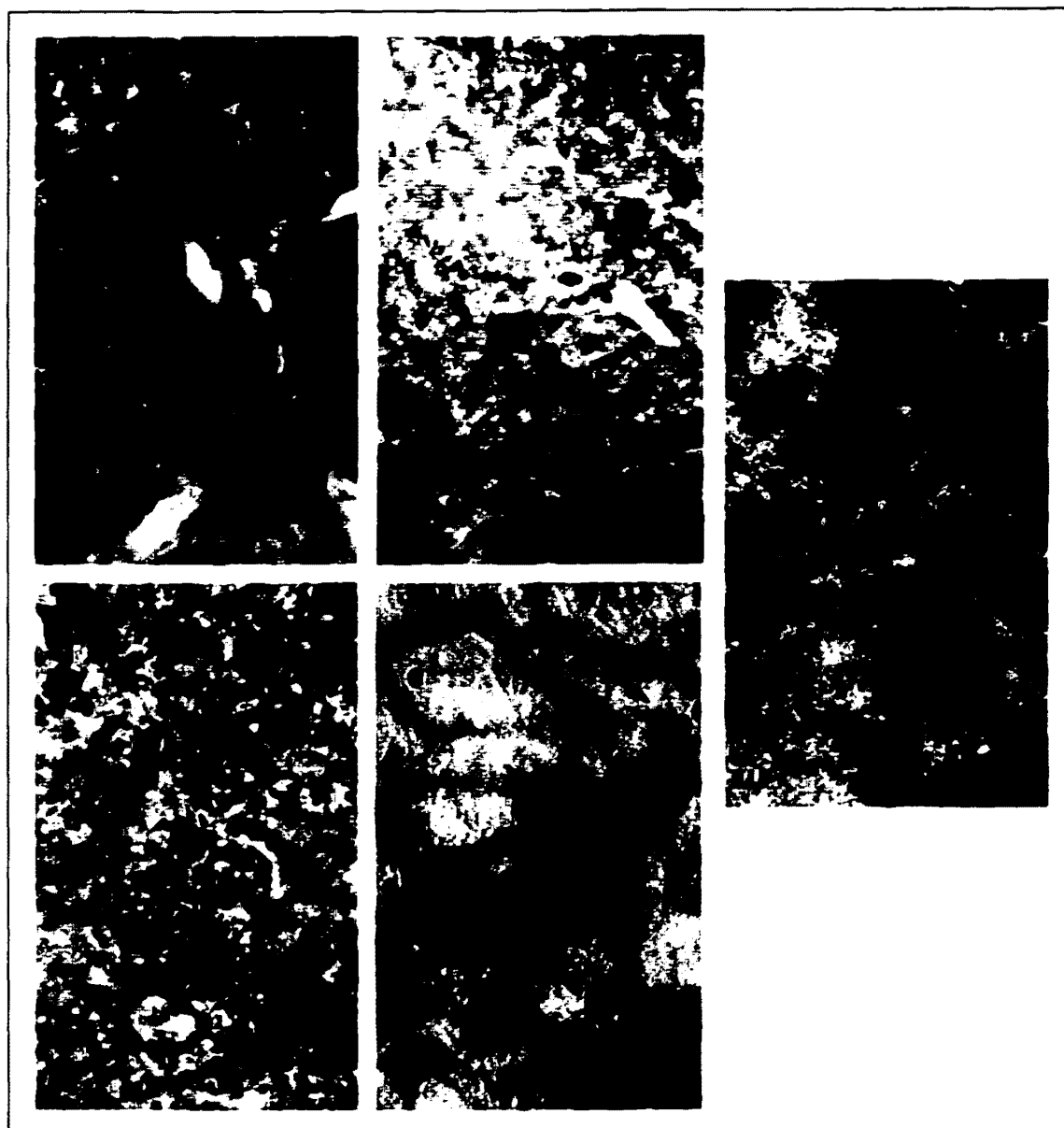
Figure 9. Non-confluent human colonic epithelial T84 cells five hours post-inoculation with B2F1 (panel A), and H30 (panel B), or DH5 α (panel C). B2F1 is intimin-negative, H30 is intimin-positive, and DH5 α is the poorly adherent control strain.



The level of adherence of all isolates to confluent monolayers of T84 cells after one hour of incubation was insufficient for microscopic detection. Three hours of incubation was required before it was apparent that a substantial number of B2F1 bacteria had bound to T84 cells, but after three hours very few H30 and 86-24 bacteria could be detected (data not shown). The intimin-positive strains took longer to form colonies but after five hours appeared to adhere well to T84 cells. After 5 hours of incubation, B2F1, 86-24 and H30 adhered to confluent cultures of T84 cells (Figure 10). In contrast, following three or five hours of incubation the *eae* deletion mutant 86-24*eae*Δ10 did not bind well to T84 cells, and only an occasional DH5α bacterium was seen attached to the monolayers (Figure 10). When confluent monolayers were used, the amount of non-specific binding appeared to be confined to the plastic slide not covered by the T84 cell monolayer; this area was typically located at the extreme periphery of the T84 monolayer. From these findings we concluded that the binding of wild-type B2F1 to T84 cells should be visually distinguishable from the binding of adherence-deficient mutants of B2F1 after three hours of incubation.

To determine if the incubation period of B2F1 on T84 cells could be shortened to one hour, the size of the bacterial inoculum was increased from a ratio of one-hundred bacteria to one T84 cell (100:1) to a ratio of 1000:1. At this 1000:1 ratio, the eukaryotic cell sheet was destroyed within the one-hour incubation period whether B2F1 or DH5α were used as the infecting organisms. Therefore, a one hour incubation period was not feasible as a means of screening mini-Tn5*phoA*Cm^r mutants for adherence defects. We also concluded that a ratio of 100 bacteria to one T84 cell would be the standard inoculum for all subsequent adherence assays.

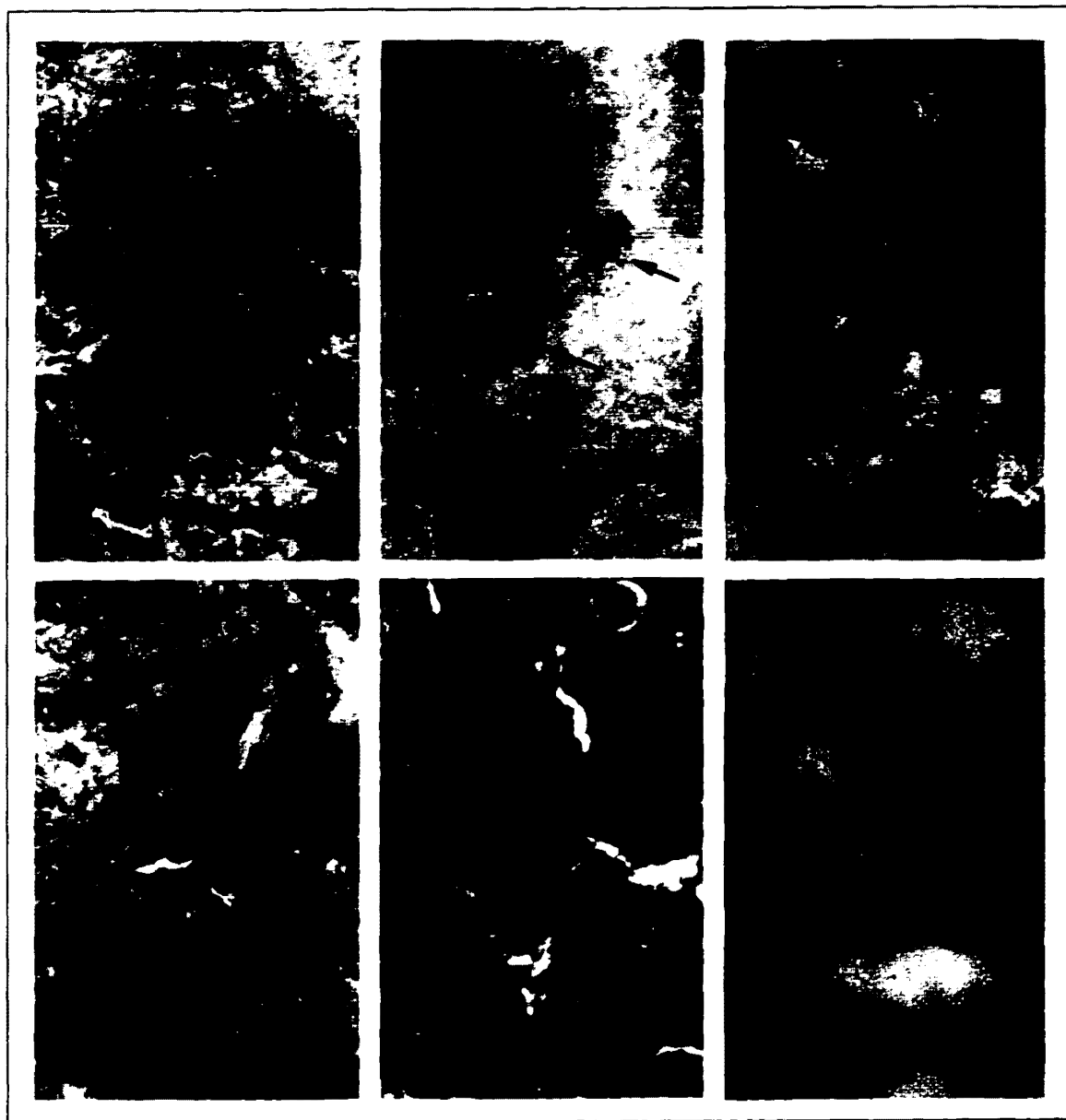
Figure 10. Confluent T84 cells infected for five hours with B2F1 and intimin-positive STEC. Strains tested were: B2F1 (panel A), EHEC 0157:H7 strain, 86-24 (panel B), and EHEC 026:H11 strain H30 (panel C) or 86-24*eae*Δ10 an *eae* deletion mutant of 86-24 (panel D), and *E.coli* DH5α (panel E).



Phenotypic comparison of the capacity of B2F1 to adhere to polarized versus non-polarized T84 cells.

Next we sought to determine if the kinetics and relative degree of binding of wild-type B2F1 to T84 cells would be the same for non-polarized and polarized T84 cells. For this purpose, polarized T84 cells that had transepithelial resistance values greater than or equal to 500 Ω were used in the adherence assay and compared to non-polarized T84 cells that had been grown to confluency on plastic chamber slides. Three strains were tested for binding: B2F1, 86-24, and DH5 α . Following a five hour incubation period, phase contrast light microscopy was used to evaluate the adherence of these organisms to polarized T84 cells. B2F1, by microscopic analysis, appeared to bind to polarized and non-polarized T84 cells in similar numbers and in the same pattern of large disorganized macrocolonies (Figure 11 panel A nonpolarized, and panel B polarized T84 cells incubated with B2F1). However, 86-24 bacteria inoculated onto polarized T84 cells formed microcolonies that were highly organized with bacteria tightly associated to each other (Figure 11 panel D). This phenotype was different from the larger less organized and less compressed 86-24 colonies associated with non-polarized T84 cells (Figure 11 panel C). Because B2F1 attaches to non-polarized and polarized T84 cells with a similar phenotype, we concluded that non-polarized T84 cells, which are easier to maintain and can be grown in plastic chamber slides, could be used in the initial screen for adherence mutants. We also decided that the more physiologically relevant polarized T84 cells would be used to confirm the reduced adherence phenotype of any mutants generated.

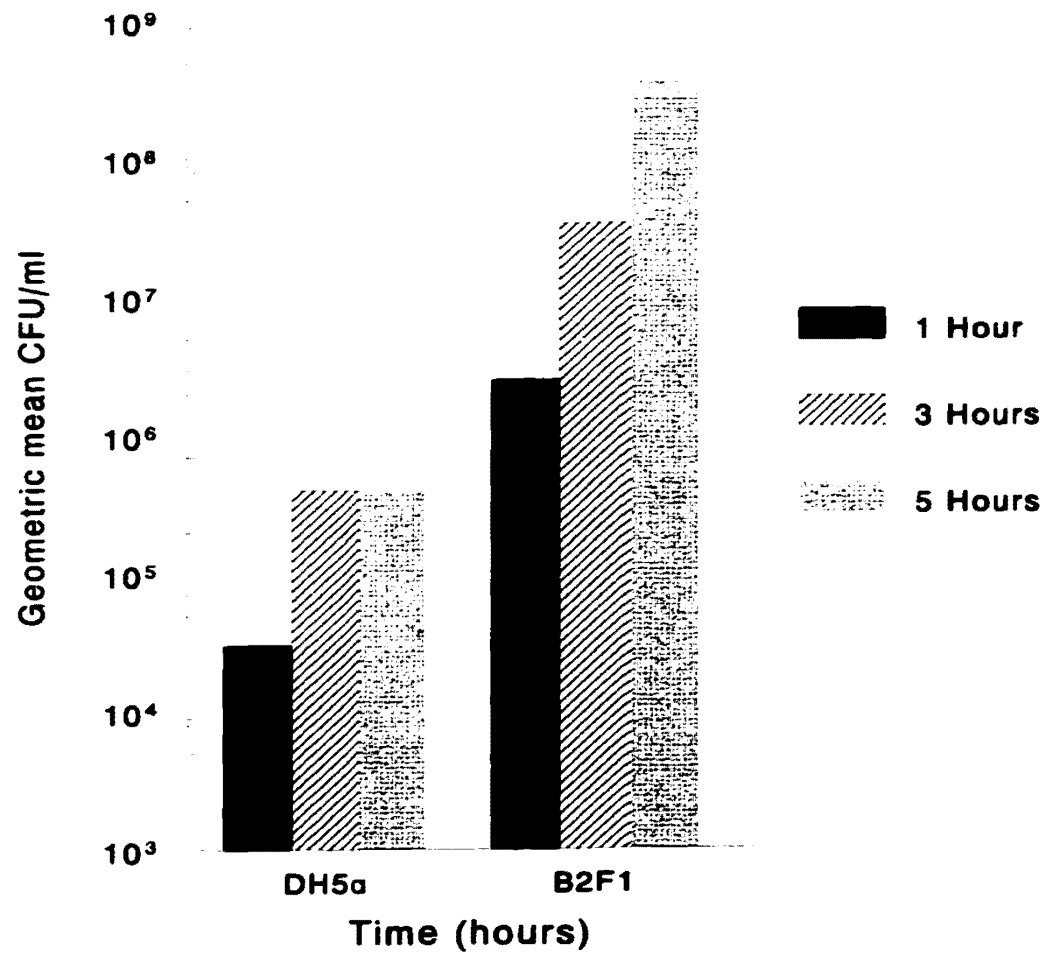
Figure 11. Polarized (panels B, D, F) and non-polarized (panels A, C, E) T84 cells inoculated with B2F1(panels A and B), 86-24 (panels C and D) or DH5 α (panels E and F) and incubated for 5 hours. Arrows indicate location of adherent bacteria.



The non-pathogenic strain of intestinal flora of *E. coli*, HS, was included in the above assay (not shown). Isolate HS avidly bound to polarized and non-polarized T84 cells in an aggregative-like fashion (data not shown). The aggregative binding pattern of HS was significantly different from that of B2F1 and of EHEC bacteria. The results suggest that T84 cells are an appropriate cell line for *in vitro* adherence analysis of intestinally adherent organisms.

Quantitative analysis of B2F1 adherence to T84 cells. To confirm the microscopic data on B2F1 binding to T84 cells, a quantitative adherence assay was developed. At the end of one, three, and five hours of incubation, non-polarized T84 cells with attached B2F1 or DH5 α bacteria were removed with trypsin from 8-cell chamber slides. The bacterial-eukaryotic cell suspension was serially diluted and plated on LB agar to obtain viable colony counts. Each time point was done in triplicate for both strains of bacteria. The geometric mean adherent colony forming units (CFU) was calculated and plotted for both of the experimental strains used (Figure 12). Differences between adherence of B2F1 and DH5 α strains that were observed microscopically were reflected as differences in the magnitude of the CFUs recovered in the representative quantitative assay. Adherence levels of STEC strain B2F1 were greater than that of the control DH5 α for all three time points tested. DH5 α values did not exceed 5×10^5 adherent CFU/ml at any time, whereas B2F1 adherent bacteria yielded a 5 hour value of 3.5×10^8 CFU/ml. As previously noted, DH5 α bacteria were typically seen attached only to the plastic slides and at the periphery of the monolayer, a finding that could account for the number of DH5 α bacteria recovered.

Figure 12. Quantitative adherence assay comparing the amounts of B2F1 bacteria and *E. coli* K-12 strain, DH5 α attached to non-polarized T84 cells. The kinetics of attachment was compared for both strains at 1, 3, and 5 hours. Geometric mean values from triplicate samples are shown for each time point.



A difference in adherence kinetics of intimin-encoding strains 86-24 and H30 to T84 cells and adherence by B2F1 to these cells had been observed by phase contrast microscopy. We theorized that if the visual data correlated with the quantitative results, mutants that were slower to bind to T84 cells could be detected. A quantitative assay with non-polarized T84 cells was done to test whether the lag phase noted for H30 and 86-24 would correlate with the number of CFU/ml recovered following a three and five hour adherence assay.

After three hours of incubation, 3×10^5 CFU/ml were recovered for H30 and 86-24 strains (Figure 13). In contrast, three hours post infection, the amount of B2F1 recovered was two logs higher at 2×10^7 CFU/ml. Microscopic analysis supported the quantitative results of the three hour post infection cultures and (photomicrographs are not shown for this time point) indicated that the levels of B2F1 bacteria bound to T84 cells were greater than levels of 86-24 and H30. After five hours of incubation with T84 cells (Figure 13), adherence levels of B2F1 were similar to that of intimin-positive strains 86-24 and H30 (3×10^8 , 8×10^7 , and 1.9×10^8 adherent CFU/ml, respectively). The number of DH5 α and 86-24*eae* Δ 10 recovered ($\sim 10^5$ CFU/ml) were 2 to 3 logs lower than that for the STEC strains at three and five hours after infection. These results indicate that the quantitative assay would be useful to detect reduced adherence mutants of B2F1 and to detect mutants that were slower to adhere to T84 cells, i.e. those mutants that adhered with different kinetics.

Quantitative results using polarized T84 cells (experiment was done in triplicate) were similar to those obtained when non-polarized T84 cells were used. B2F1 adherence values were 7×10^7 CFU/ml after 5 hours in contrast to 10^3 CFU/ml of DH5 α (Figure 14). Because the polarized monolayer of T84 cells extended completely across the collagen-coated membrane

Figure 13. Comparison of the number of intimin-encoding STEC (86-24 and H30) and intimin-negative B2F1 adherent to non-polarized T84 cells three and five hours post-infection. Geometric mean values from triplicate samples are shown for each time point.

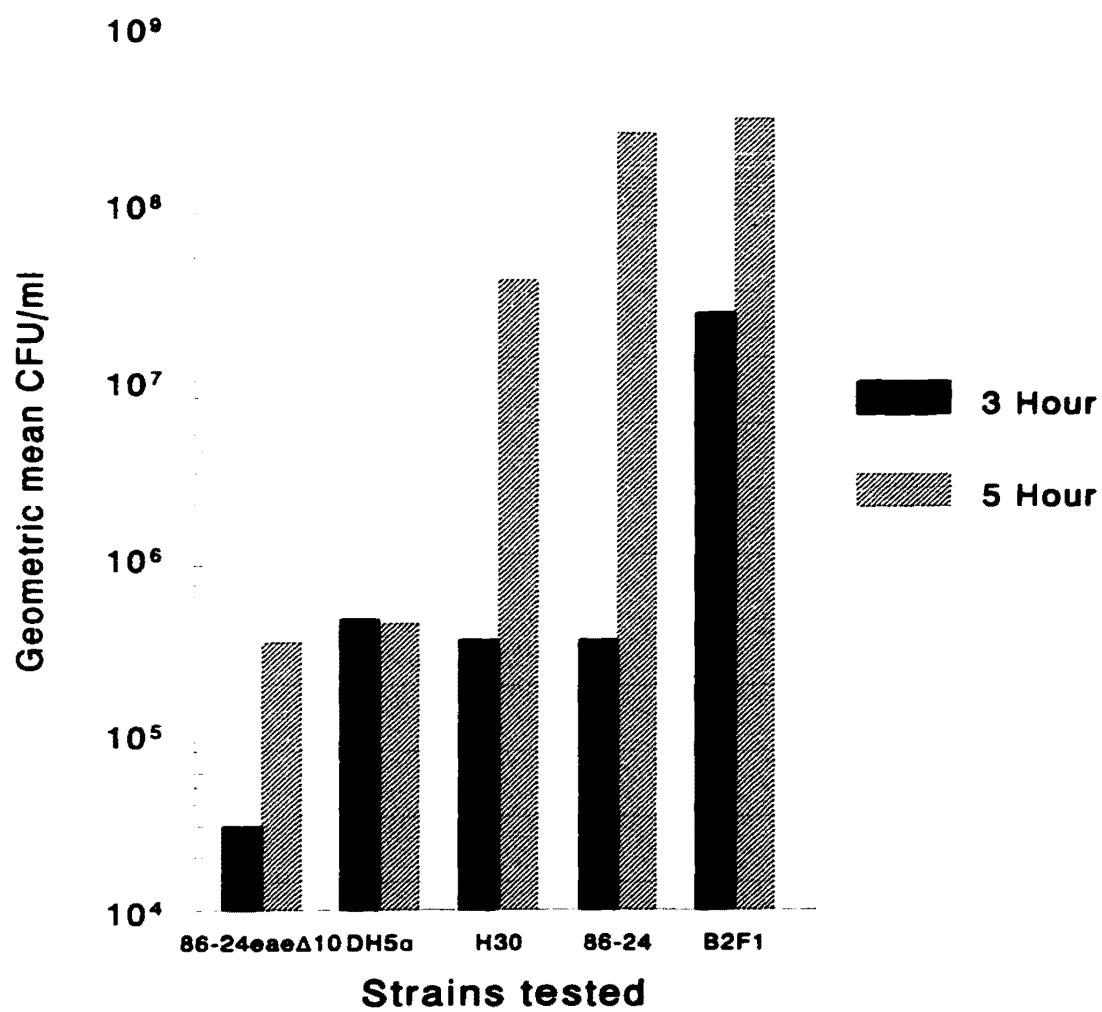
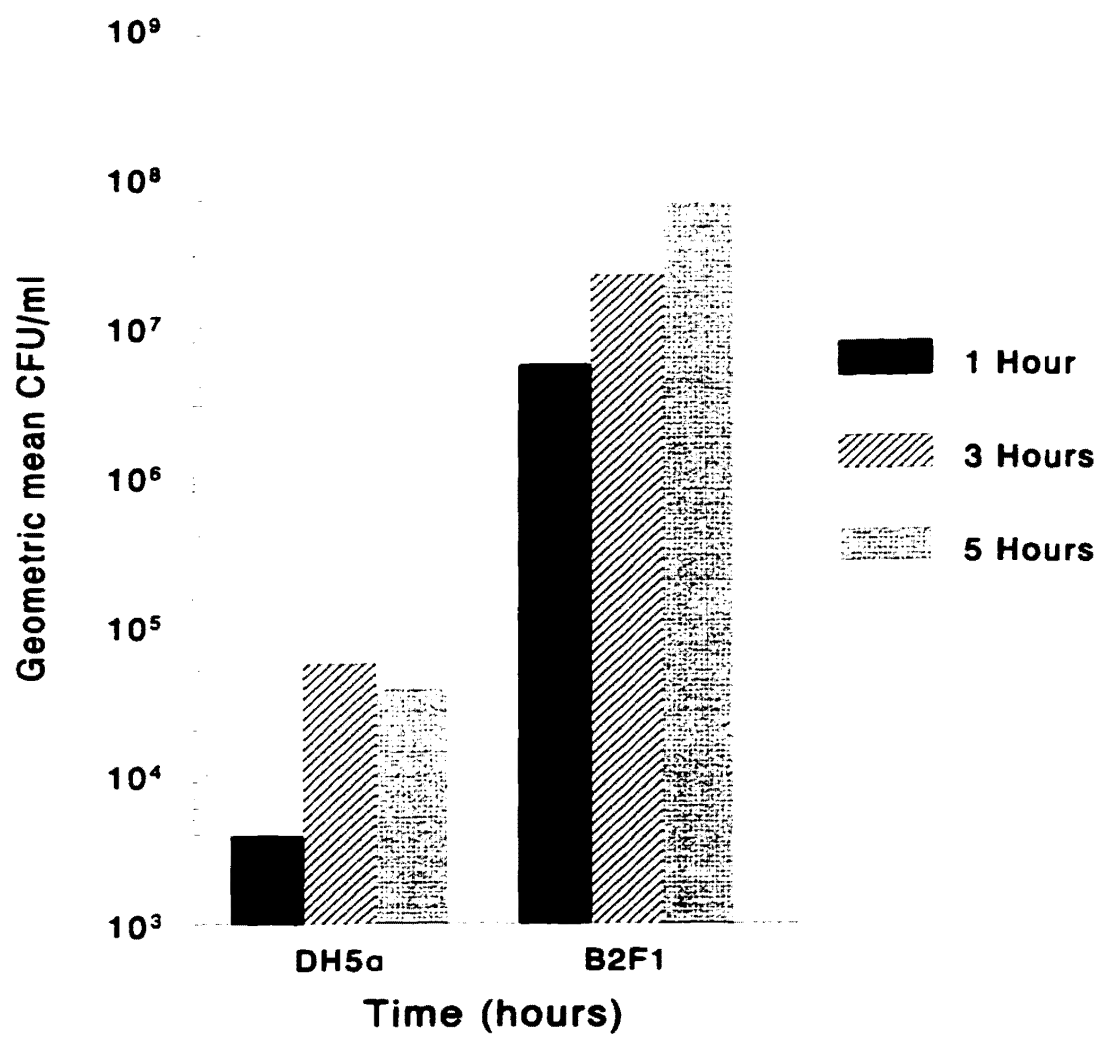


Figure 14. Quantitative adherence assay that compared the number of B2F1 bacteria adherent to polarized T84 cells versus the poorly adherent *E.coli* K-12 strain DH5 α . The amount of bacteria recovered was compared 1, 3, and 5 hours post-inoculation. Geometric mean values of triplicate samples are shown for each time point.



with no discernible gaps, the amount of non-specific binding of DH5 α was reduced. However, the CFU/ml of bacteria recovered for B2F1 were comparable to those obtained when non-polarized monolayers were used. By comparison of the quantitative data obtained with polarized versus non-polarized T84 cells, we concluded that the easy to use non-polarized T84 cells could be used to confirm quantitatively the microscopic evaluation of differences in adherence of mini-Tn5*phoAC*^r isolates and wild-type B2F1 to T84 cells but that the polarized T84 cells would be used as the ultimate standard to more precisely define quantitative differences in adherence to T84 cells between B2F1 and putative adherence mutants. Therefore we elected to use the scheme outlined in Figure 15 to screen and test putative adherence mutants of B2F1.

III. The role of the large ~90 kb plasmid in the adherence to human colonic epithelial T84 cells by STEC B2F1.

The experiments described below were designed to determine if the large plasmid encoded adherence factors, or if its presence was necessary for adherence of STEC bacteria to human intestinal epithelial cells.

Transformation of a poorly-adherent laboratory *E. coli* strain DH10B with the large plasmid isolated from an isogenic mutant of strain B2F1. An isogenic mini-Tn5*phoAC*^r mutant of B2F1 was isolated in this laboratory prior to this study and was designated as strain 2B3. Strain 2B3 contained a mini-Tn5*phoAC*^r insertion within the large ~90 kb plasmid. Because hemolysin is encoded on the large ~90 kb plasmid of STEC bacteria, strain 2B3 was hemolytic on washed sheep red blood cells. Strain 2B3 was also

Figure 15. Schematic of the *in vitro* adherence assay used to evaluate attachment of STEC strains and mini-Tn5*phoA*Cm^r mutants. The parameters of the assay are discussed in detail in the text.

Screen Mini-Tn5*phoA*Cm^r mutants in a 3 hour adherence assay with **non-polarized** T84 cells. Assess visually.



Re-evaluate putative adherence mutants for capacity to adhere to **non-polarized** T84 cells after 5 hours of incubation. Assess visually.



Retest adherence-defective mini-Tn5*phoA*Cm^r mutants in a 3 hour **quantitative** adherence assay with **non-polarized** T84 cells.

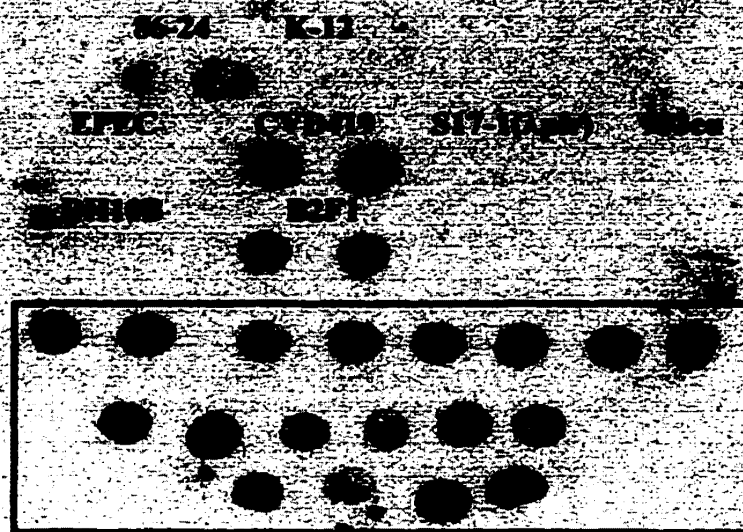


Confirm reduced adherence phenotype of mini-Tn5*phoA*Cm^r mutants in a 3 hour **quantitative** adherence assay with **polarized** T84 cells.

chloramphenicol resistant by virtue of the presence of the mini-Tn5*phoA*Cm^r insertion within the large plasmid. The large plasmid (p2B3) was purified from 2B3 and electroporated into a non-pathogenic, *E. coli* laboratory strain, DH10B. Chloramphenicol-resistant transformants were tested for the presence of the ~90 kb plasmid by colony hybridization with probe CVD419 (Figure 16). One probe-positive transformant, DH10B(p2B3) was selected for further study. Strains DH10B, DH10B(p2B3), 2B3 and wild-type B2F1 were then tested in the standard three and five hour qualitative adherence assays to assess whether they attach to T84 cells. Strain 2B3, attached to T84 cells at levels comparable to its parent B2F1, (5 hour results shown in Figure 17 panel A and B, respectively) and as expected, the laboratory strain DH10B did not adhere to T84 cells (Figure 17 panel D). Because the isogenic mutant 2B3 adhered as well to T84 cells as did wild-type, it was presumed that the transposon had not inserted within a gene that encoded factors essential for adherence (Figure 17 panel A). The transformant, DH10B(p2B3), adhered poorly to T84 cells; similar to the parental DH10B strain (Figure 17 panel C and D). In conclusion, this experiment indicates that the ~90 kb plasmid derived from STEC 2B3 is not in itself sufficient to impart the wild-type adherence phenotype to a previously poorly adherent laboratory strain of *E. coli*, DH10B.

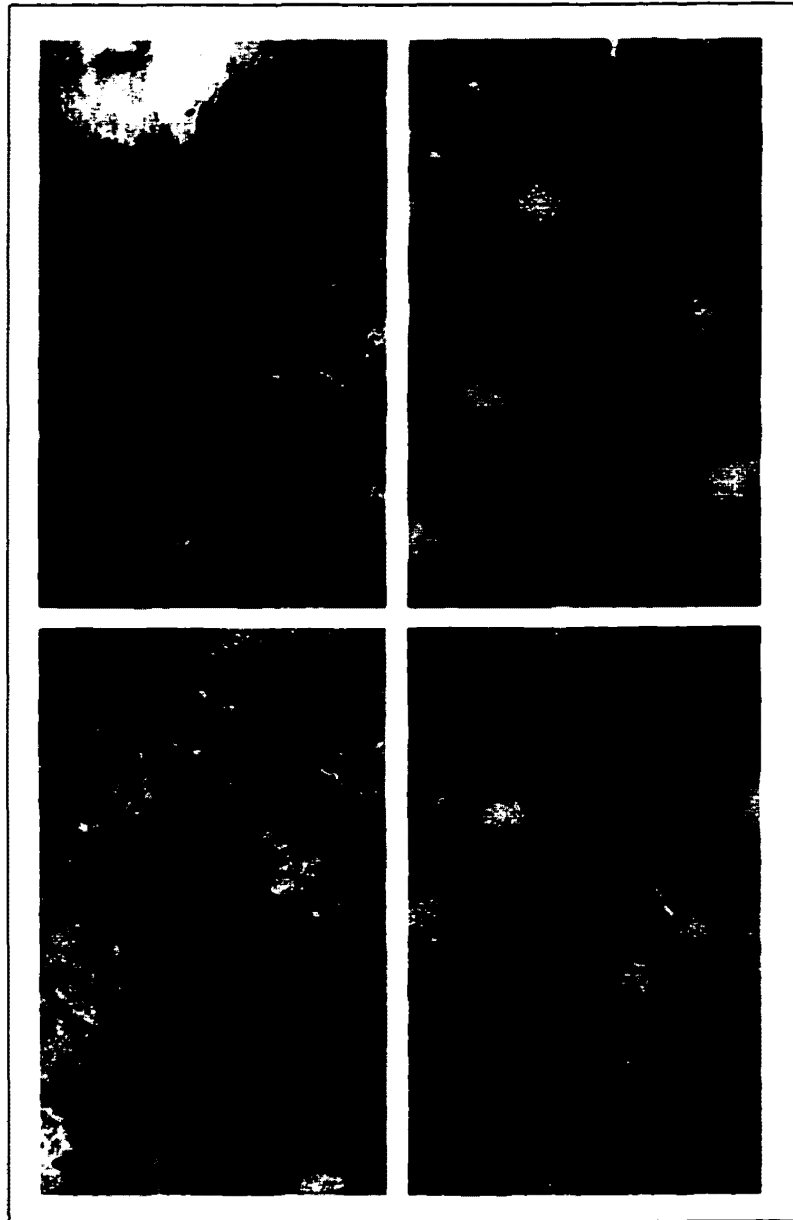
Capacity of a plasmid-cured derivative of B2F1 to bind to colonic epithelial T84 cells. A derivative strain B2F1 devoid of the large plasmid was isolated previously by others in this laboratory. This plasmid-cured derivative was designated as S11, and, due to the absence of the large plasmid, it was hemolysis negative on washed sheep red blood cells.

Figure 16. *E. coli* DH10B(p2B3) transformants tested by colony blot hybridization with probe CVD419. Probe CVD419 consists of a 3.4 kb *Hind*III fragment derived from plasmid p0157 carried by STEC strain 933. Negative control strains included the parental strain DH10B, 933cu, EPEC, *E. coli* K-12 strains DH5 α and S17-1(λ pir). Positive controls consisted of strains 86-24, B2F1 and probe CVD419 DNA.



DH10B (p2B3) transformants

Figure 17. Human colonic epithelial T84 cells five hours post-infection with strain 2B3 (panel A), B2F1 (panel B), transformant DH10B(p2B3) (panel C) and the parental strain DH10B (panel D).



The absence of the plasmid was confirmed by colony blot analysis with the plasmid specific probe CVD419 (Figure 18). Colonies of EHEC 0157:H7 (86-24 and 933) and parental strain B2F1 served as positive controls, while *E. coli* strain DH10B and the plasmid-cured derivative of 933 were the negative control strains used in this colony blot.

After five hours the plasmid-cured derivative, S11, attached to T84 cells as well as the parental wild-type strain (Figure 19). Quantitative analysis was done using non-polarized T84 cells and indicated that there was no significant difference in adherence to T84 cells between B2F1 and S11 (data not shown).

IV. Attempt to construct by P1 transduction an alkaline phosphatase null mutation in B2F1.

Wild-type B2F1 is naturally phosphatase positive and produced blue colonies when grown on LB agar that contained XP. Hence, generalized P1 transduction was used to try to transfer a non-functional *phoA* deletion mutation encoded by the *E. coli* strain SH210 into wild-type B2F1. The *phoA* mutation in SH210 is linked to Tn10 that encodes the tetracycline resistance gene. The transduction procedure that was used is detailed in Materials and Methods. Bacteriophage P1::Tn9_(SH210) encodes a temperature-sensitive repressor that at 42 °C becomes inactive. A shift in temperature from 37°C to 42°C induced the bacteriophage to become lytic. The Tn9 insertion within this P1 phage carries a chloramphenicol resistance gene that serves as a selectable marker. A high titer lysate (~1 x 10¹¹ PFU/ml) of P1::Tn9_(SH10) was prepared and was used in an attempt to infect B2F1. Simultaneously, a control laboratory strain C600 was transduced with the same amount and sample preparation of lysate. Forty per cent of the isolates

Figure 18. Colony hybridization of the plasmid-cured derivative of B2F1, designated as S11 with probe CVD419. EHEC strains 933, 86-24JBK, 86-24m, 86-24, H30, and STEC strain B2F1 contained the ~90 kb plasmid and served as the positive controls. Strains 933cu and DH5 α . were the negative controls.

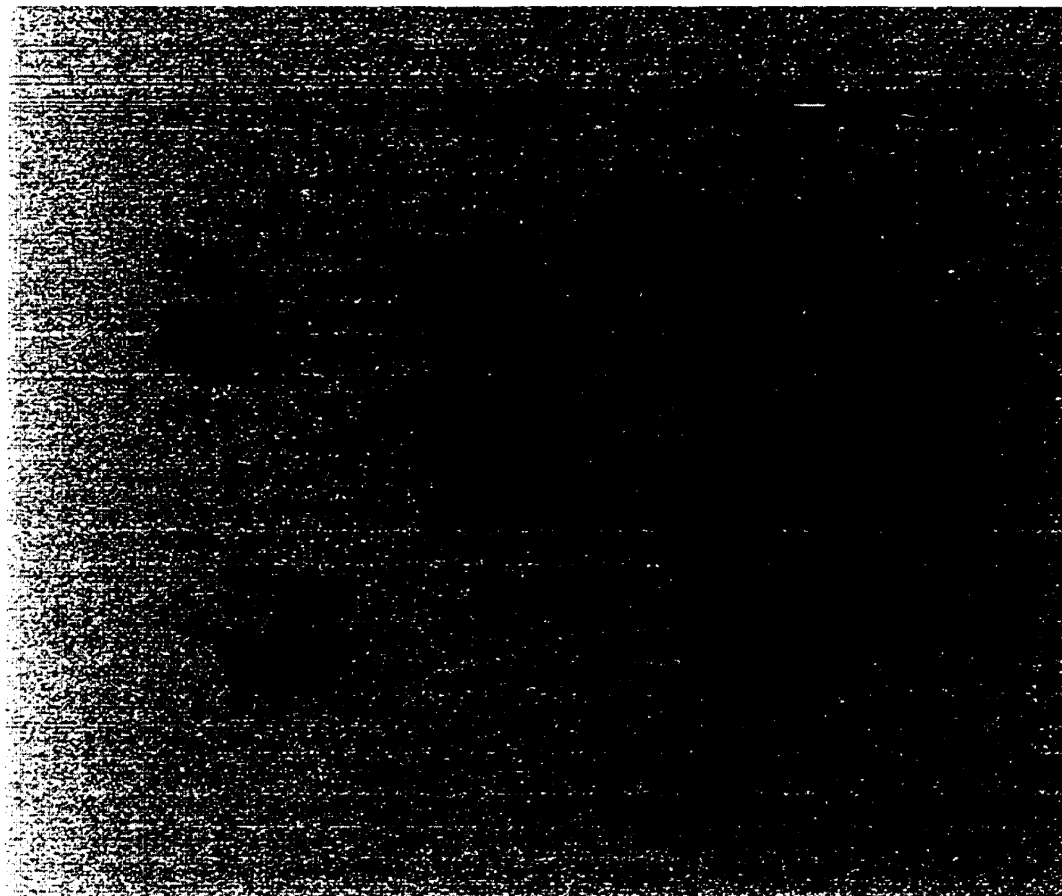
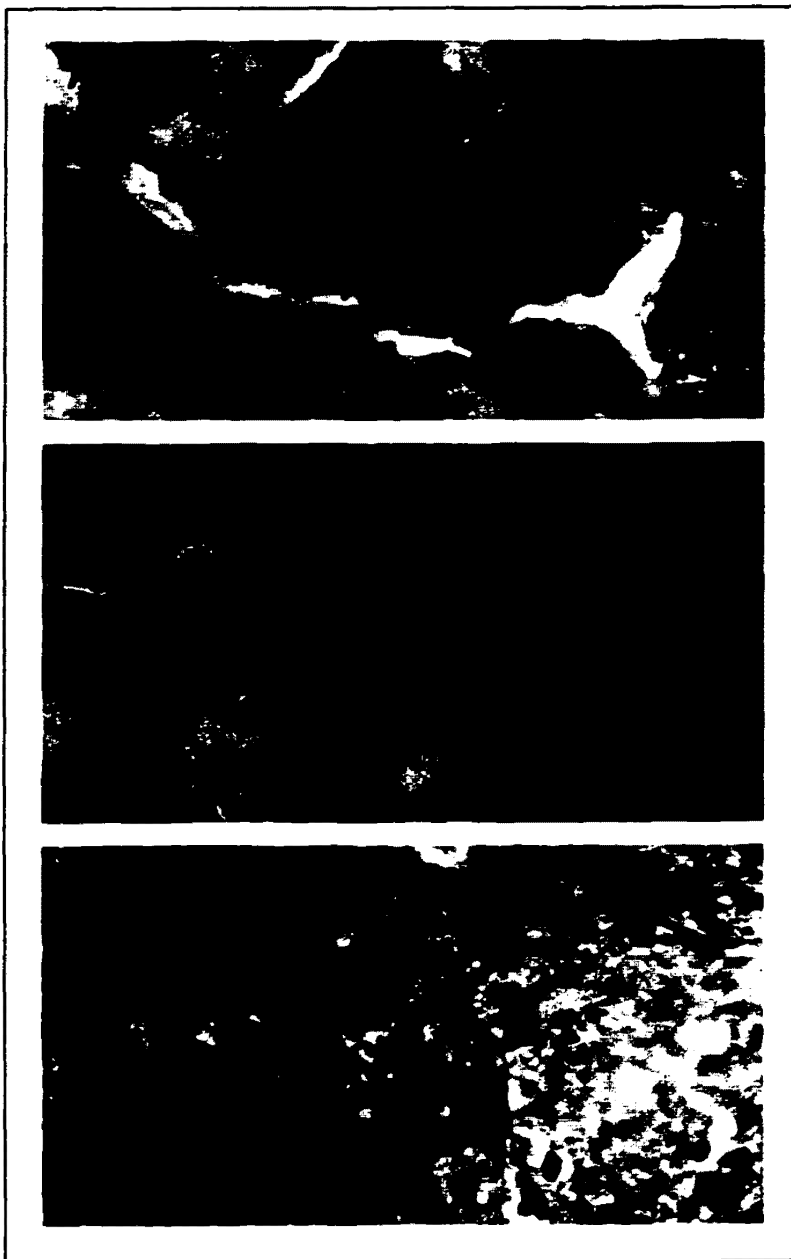


Figure 19. Comparison of wild-type B2F1 and its plasmid-cured derivative S11 to adhere to T84 cells five hours after infection. Strains tested: S11 (panel A), B2F1 (panel B), and DH5 α (panel C).



were cotransduced and contained both the *phoA* mutation and the Tn10 encoded tetracycline resistance gene from strain SH210. However, P1::Tn9 could not infect B2F1 even when used at a multiplicity of infection of one-hundred bacteriophage to one bacterium (no chloramphenicol or tetracycline resistant colonies were isolated). Therefore, an alternate approach was used to facilitate discrimination between wild-type B2F1 and putative in frame, blue mini-Tn5*phoA*Cm^r mutants of that organism.

V. Construction and screening of the mini-Tn5*phoA*Cm^r mutant bank prepared from wild-type strain B2F1.

Isolation of mini-Tn5*phoA*Cm^r mutants. When 80 µg/ml of XP was added to LB agar, the blue phenotype of wild-type B2F1 took longer than 18 hours to develop. In contrast, isolates of B2F1 with in-frame insertions of mini-Tn5*phoA*Cm^r within genes encoding exported or surface exposed proteins could be detected within 13 to 15 hours after plating. Because it took longer for wild-type colonies to turn blue, mutants with *phoA* fusions could be differentiated from wild-type if the blue color of colonies were scored within 13 to 15 hours. After this initial screen, mutants were re-streaked for isolation, frozen, and tested for the blue phenotype on LB agar that contained forty micrograms per milliliter of XP.

After numerous matings between the mini-Tn5*phoA*Cm^r host strain S17-1(*λpir*) and B2F1, 22,555 transconjugates were isolated. Because mating was allowed to occur overnight in some cases, some of the transconjugates that were isolated may have been siblings. There were 253 isolates that produced blue colonies faster than wild-type (within 15 hours) and that were a darker blue than wild-type. These isolates were presumed to contain in-frame insertions

in genes that encoded exported proteins and represented approximately one percent of the total transconjugates isolated (Table 5). All isolates that were blue on XP-LB agar (253 total) were screened for adherence to T84 cells. Only 5 of the 253 blue isolates (2%) were ampicillin resistant; the other 248 colonies were ampicillin sensitive.

Isolation of three adherence-deficient mutants of STEC 091:H21 strain

B2F1. Three mutants designated as 34.3, 34.7 and 30.10a were blue on XP-LB agar (see Figure 20), chloramphenicol resistant, and ampicillin sensitive. Thus, they were presumed to be cured of the donor plasmid that carried the mini-Tn5*phoA*Cm^r cassette. These three mutants grew at rates similar to wild-type B2F1 when grown in Knutton tissue culture adherence assay media at 37°C (Figure 21). Three of the 253 isolates that contained the transposon were reduced in their capacity to bind to T84 cells (Figures 22 and 23).

The isolates, 34.3, 34.7 and 30.10a, were tested in the standard three and five hour qualitative adherence assay with nonpolarized T84 cells. Assays were done in triplicate on more than three separate occasions, and several isolates of each mutant were tested. Microscopic evaluation of mutants 34.3 and 34.7 (Figure 22 panel B and C results of a representative 5 hour assay) indicated that both strains failed to adhere well to T84 cells.

Mutant 30.10a did not adhere well to T84 cells, (Figure 22 panel A) and the pattern of the limited number of adherent organisms was distinct from wild-type. Specifically, strain 30.10a attached as individual bacteria with several bacteria attached to and suspended above the cell sheet of non-polarized T84 cells (Figure 19 panel A) or polarized (not shown). In contrast, B2F1 adhered in large colonies to T84 cells (Figure 22 panel E). The total CFU/ml recovered three hours after infection of polarized T84 cells were compared for strains 34.3,

Table 5. Results of conjugal matings for transfer of mini-Tn5*phoACm^r* to create a mutant bank of STEC 091:H21 strain B2F1

Duration of mating	No. transconjugates isolated	No. of blue colonies (PhoA ⁺)
one hour	7,592	94
18 hours	14,963	159
	22,555	253

Figure 20. Appearance of mini-Tn5*pho*ACm^r mutants designated as 34.3, 34.7 and 30.10a and parental strain B2F1 grown on XP agar. Strains were grown for 15 hours on agar supplemented with 80 µg/ml of the alkaline phosphatase substrate XP.

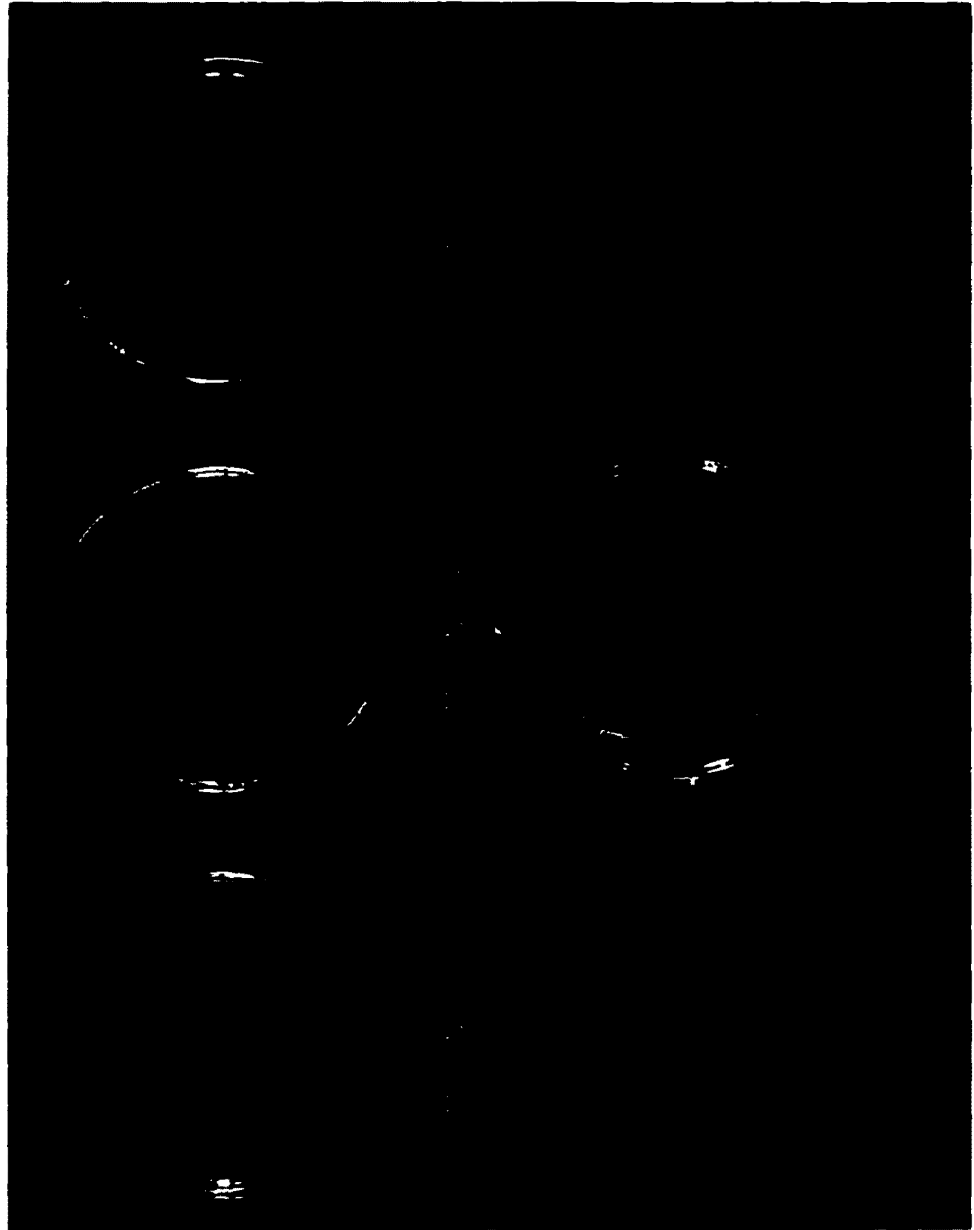


Figure 21. Growth rates of three adherence mutants, 34.3, 34.7 and 30.10a, compared with parental strain B2F1 when grown in Kutton tissue culture adherence assay media at 37°C. Cultures were incubated for a total of 8 hours with shaking and the optical density 600 nm was recorded every hour. The initial reading through 6 hours are shown on the graph.

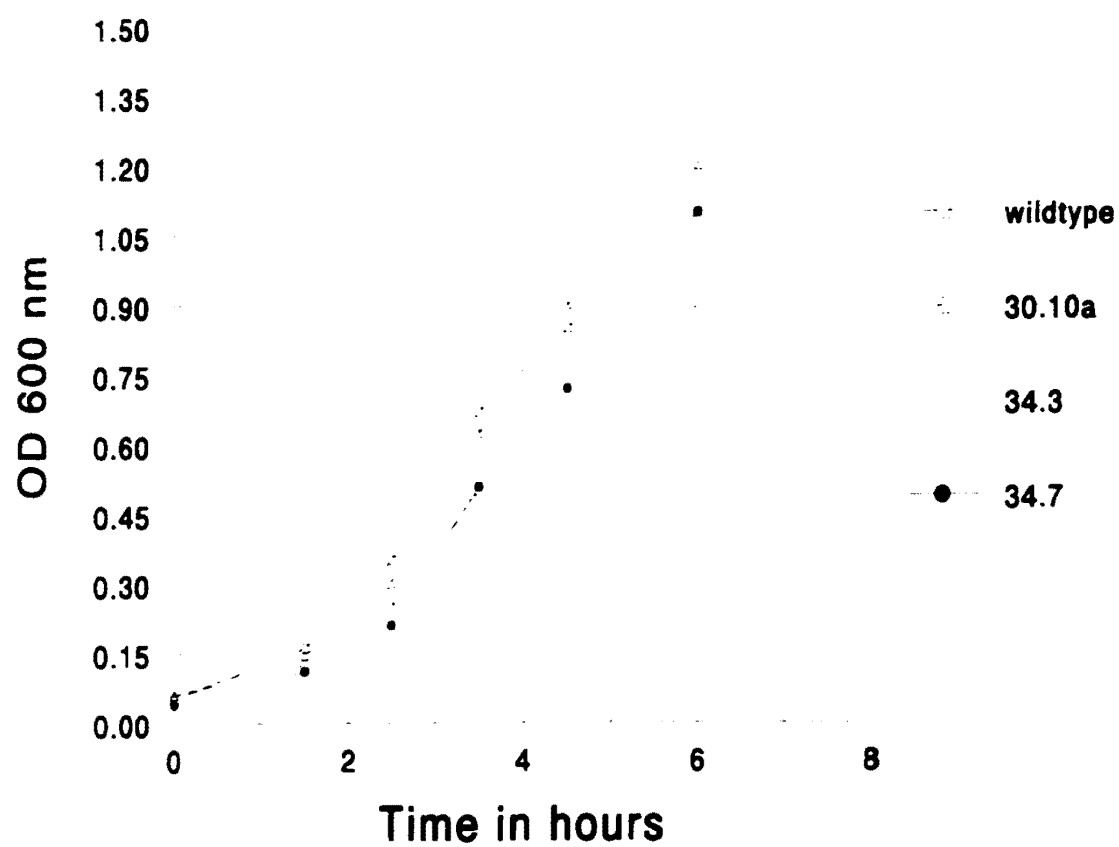


Figure 22. Capacity of mini-Tn*5phoA*Cm^r mutants 30.10a (panel A), 34.3 (panel B), 34.7 (panel C) to adhere to T84 cells after 5 hours of incubation as compared to wild-type B2F1 (panel E). Poorly adherent control strain DH5α is shown in panel D. Arrow in panel A points to single adherent bacterium; arrow in panel E indicates a colony of organisms.

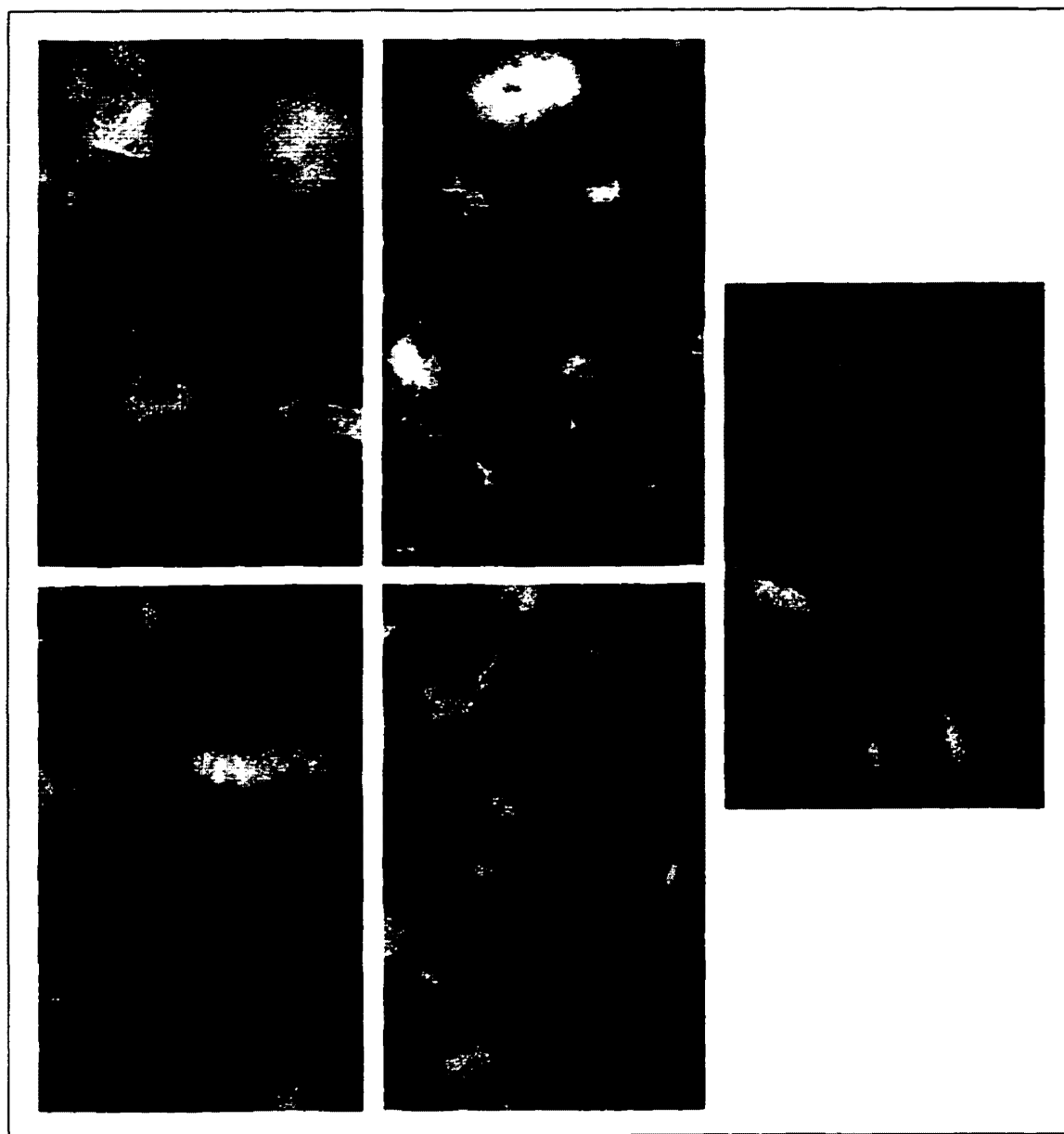
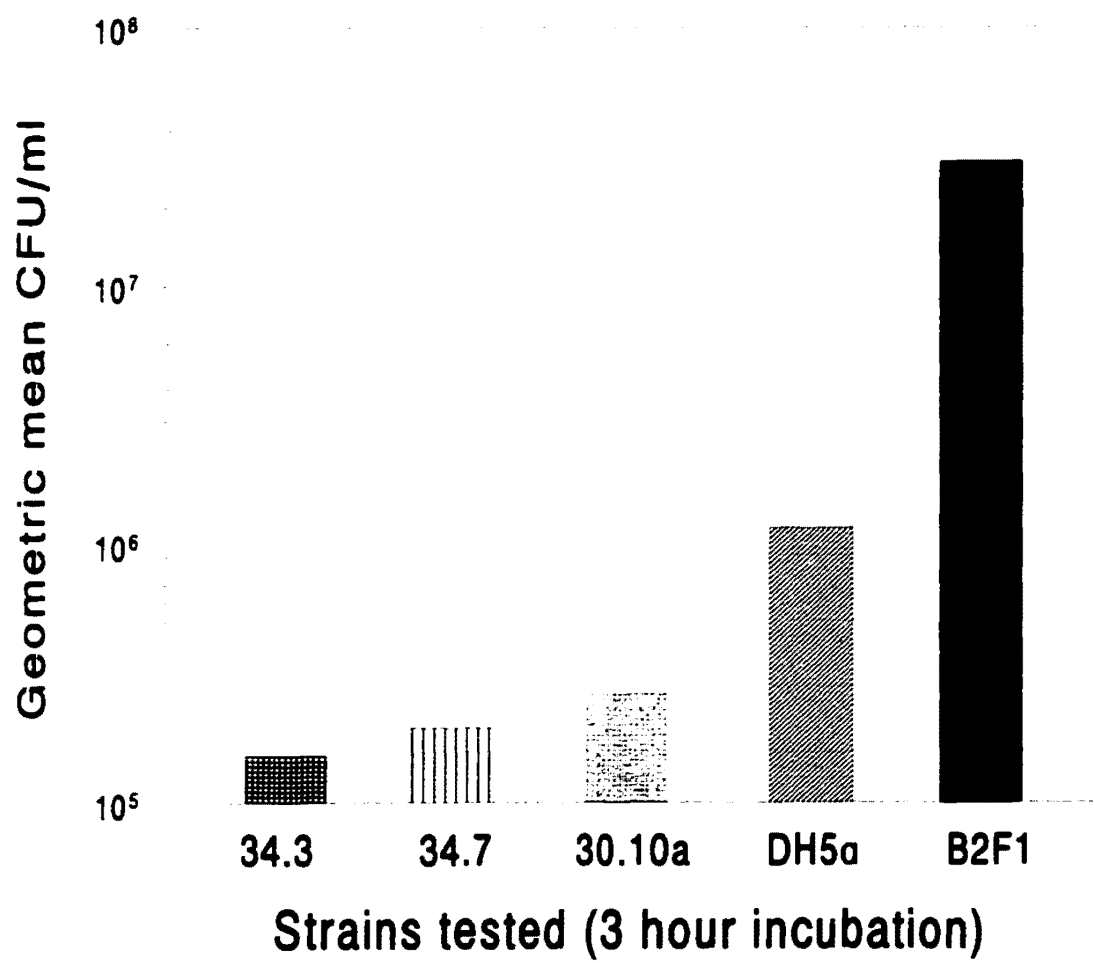


Figure 23. The number of bacteria adherent to polarized T84 cells after three hours of incubation determined for three mini-Tn5*phoAC*^r mutants, 34.3, 34.7 and 30.10a and compared to wild-type B2F1. DH5α was included as a poorly adherent control. Geometric mean values for triplicate samples are depicted for each time point.



34.7, and 30.10a to those of the parental strain B2F1 (Figure 23) [quantitative results of a three hour assay with nonpolarized T84 cells is not shown]. Approximately 10^5 CFU of each strain, 34.3, 34.7 and 30.10a, were recovered as compared to 10^6 DH5 α and approximately 10^7 of wild-type. These results suggest at least a 100-fold difference in the capacity of each of these three mutants to adhere to T84 cells as compared to the wild-type.

Following a five hour adherence assay, mutant 30.10a destroyed the T84 cell monolayer. This destruction did not always occur and appeared to depend on the age of the monolayer that was used (10-12 day old monolayers were most susceptible) [data not shown]. Mutants 34.3 and 34.7 caused no discernible, or only moderate damage, five hours after inoculation when 10-12 day old non-polarized T84 monolayers were used (data not shown).

Characterization of adherence mutants. Table 6 summarizes the phenotypes examined for each mutant. The procedures used to assess each of these characteristics has been described in the Materials and Methods section of this thesis.

Antiserum against serotype 091 bacteria agglutinated strains 34.3, 34.7 and 30.10a as well as the parental strain B2F1; hence the mutants were all smooth and of the 091 serogroup. The adherence mutants, like wild-type, produced capsule when grown on Maloney media and were not auxotrophic since they grew well on minimal M9 media. Strains 34.3, 34.7 and 30.10a grew well on MacConkey media and were not sensitive to the bile present in this medium. Strains 34.3, 34.7 and 30.10a secreted Stx2d in amounts similar to those secreted by B2F1 (quantitation of Stx2d not shown) and were all motile at 37°C. However, 34.3 and 34.7 were not motile in soft-agar when grown at 30°C whereas the parent B2F1 and mutant 30.10a were motile (data not shown). Note that the motility findings at 30°C were made subsequent

Table 6. Characteristics of adherence mutants 34.3, 34.7, and 30.10a compared to the parental strain B2F1

Strains tested	091 serogroup	Capsule	Motility		Growth on M9	Growth on MacConkey	Ampicillin sensitive	Stx detected in filtrates	Hemolysin produced
			30°C	37°C					
Wildtype	+	+	+	+	+	+	+	+	+
34.3	+	+	-	+	+	+	+	+	+++
34.7	+	+	-	+	+	+	+	+	+++
30.10a	+	+	+	+	+	+	+	+	++

to the initial phenotypic characterization of the mutants. In addition, all of the adherence mutants (34.3, 34.7 and 30.10a) produced large clear zones of hemolysis on agar plates that contained washed (Figure 24) or unwashed (not shown) sheep red blood cells (SRBC). The phenotype exhibited by the mutants was in contrast to that of B2F1 which produced small turbid zones of hemolysis visible when the SRBCs were washed before incorporation into the agar.

One possible reason for the hyper-hemolytic phenotype of the 34.3, 34.7 and 30.10a was that the outer membranes of these mutants were leaky. Such a leaky phenotype might explain the damage to T84 cells sometimes seen when these mutants were tested in the five hour adherence assay. Therefore, two tests were done to determine if the membranes of the mutants exhibited normal function e.g. maintained barrier function and if they differed in release of proteases. Strains 30.10a, 34.3 and 34.7 were grown separately in the presence of crystal violet and on LB agar plates that contained 3% instant non-fat dry milk (source of casein). Both the wild-type and the mutant strains were able to exclude the crystal violet and to grow up to the crystal violet-impregnated filter (data not shown). Similarly, none of the colonies, mutants or wild-type, produced zones of clearance on agar plates that contained casein (Figure 25). Therefore, no differences in protease leakage/export was evident among the strains. These findings taken together with the capacity of the mutants to grow in the presence of bile salts in MacConkey agar indicated that the outer membranes of isolates 34.3, 34.7 and 30.10a probably function normally. Therefore, the reason for the large, clear zones of hemolysis on SRBC agar was due to another factor(s).

VI. Restriction mapping of the mini-Tn5*phoAC*m^r insertion present in adherence mutants 34.3, 34.7 and 30.10a.

Figure 24. Comparison of the hemolytic phenotypes of wild-type B2F1, its isogenic mini-*Tn5phoACm'* adherence-defective mutants, and the plasmid-cured strain S11. Because S11 no longer carries the large plasmid, it serves as the negative control for hemolysis. Agar plates that contained 5% washed sheep red blood cells, with 10 mM CaCl added were incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. The next day, after a short 2 hour incubation at 4°C, the hemolysis phenotype was scored.

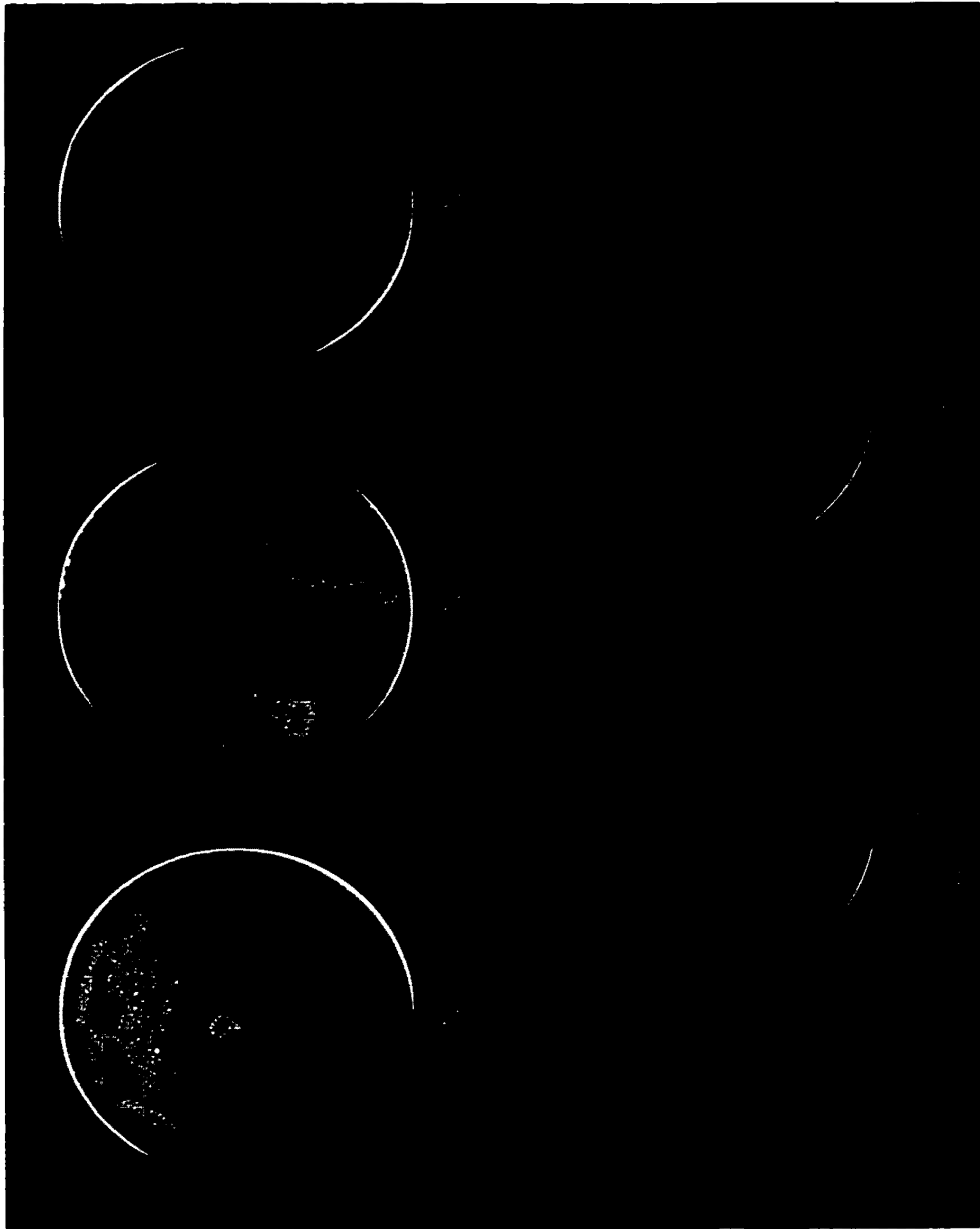
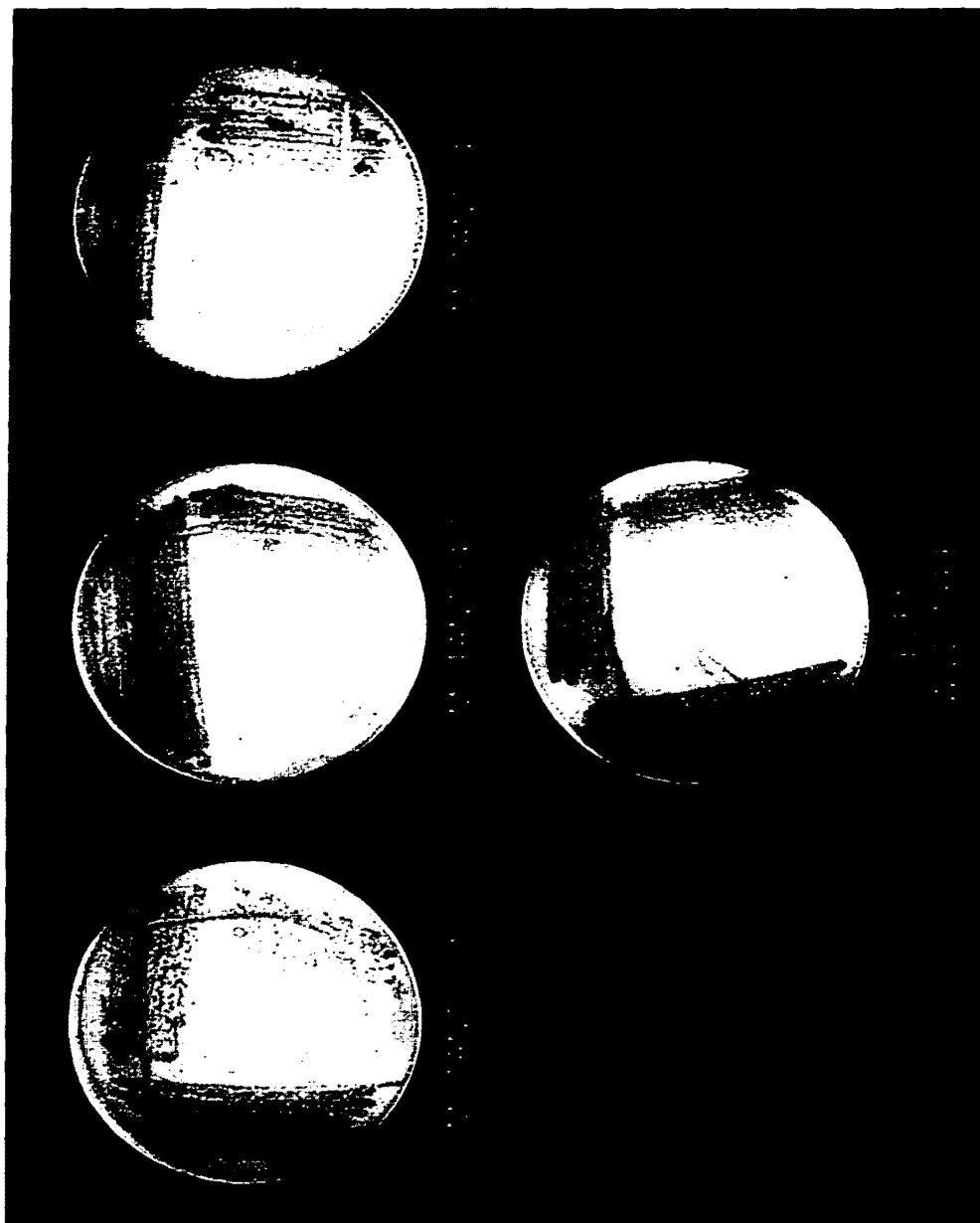


Figure 25. Comparison of the growth of B2F1 and adherence mutants 34.3, 34.7 and 30.10a on LB agar that contained 3% instant nonfat dry milk. Plates were incubated overnight at 37°C.



Southern hybridization analysis of *HindIII*-restricted total DNA isolated from mutant 34.3 and from mutant 34.7 revealed a 6 kb size fragment that hybridized to the chloramphenicol resistance gene (Cm^r) (Figure 26). Because the same size restriction fragment hybridized to the chloramphenicol probe, the possibility existed that both mutants contained the transposon insertion within the same gene. Therefore, a second set of Southern analyses was done with *Sall*-and *PstI*-singly and doubly-restricted total DNA from 34.3 and 34.7. The blots were again probed with the Cm^r (Figure 27). B2F1 served as the negative control in these experiments because it does not contain the Cm^r . The DNA hybridization pattern was the same for both mutants. Also, an identical 1.4 kb fragment was detected whether DNA was restricted with *PstI* alone or in combination with *Sall*, a finding that indicated that the *PstI* fragment was inside the *Sall* fragment for both mutants. Lastly, the size of the *Sall* DNA fragment that hybridized with the probe was 9 kb in both samples. Adherence mutants 34.3 and 34.7 were isolated from the same conjugal mating mixture, and had the same Southern hybridization pattern, thus, we concluded that 34.3 and 34.7 contained the insertion of the transposon in the same gene and were probably of the same clonal derivative.

By contrast, we concluded that mutant 30.10a contained the mini-*Tn5phoACm^r* insertion at a site distinct from that of 34.3 and 34.7 based on Southern analysis (Figure 26 and 27). DNA restricted with *HindIII* hybridized with the Cm^r probe to yield a 12 kb size fragment. Restriction of 30.10a DNA with a combination of *Sall* and *PstI* hybridized to the probe to yield a 5 kb fragment, and restriction with *PstI* alone yielded an 8 kb fragment. There is a fragment that is >23 kb present on both the *HindIII* and *Sall* Southern blot figures and in both cases this fragment probably represents incompletely cut DNA.

Figure 26. Southern hybridization of *Hind*III-restricted total DNA from B2F1 mini-Tn5*phoA*Cm^r adherence-defective mutants 34.3, 34.7, 30.10a. The chloramphenicol resistance gene from mini-Tn5*phoA*Cm^r was labeled with ECL-horseradish-peroxidase and used as the probe in the analysis. Position of molecular weight size markers (kb) are indicated.

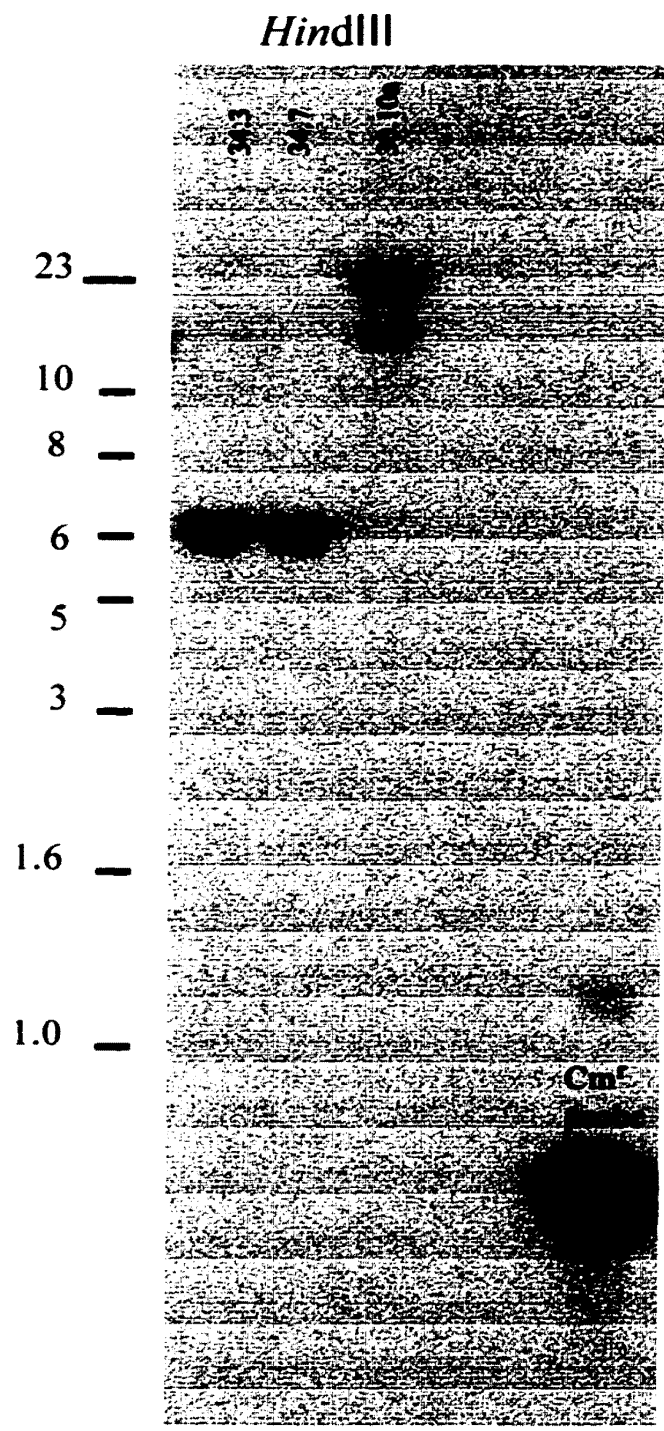
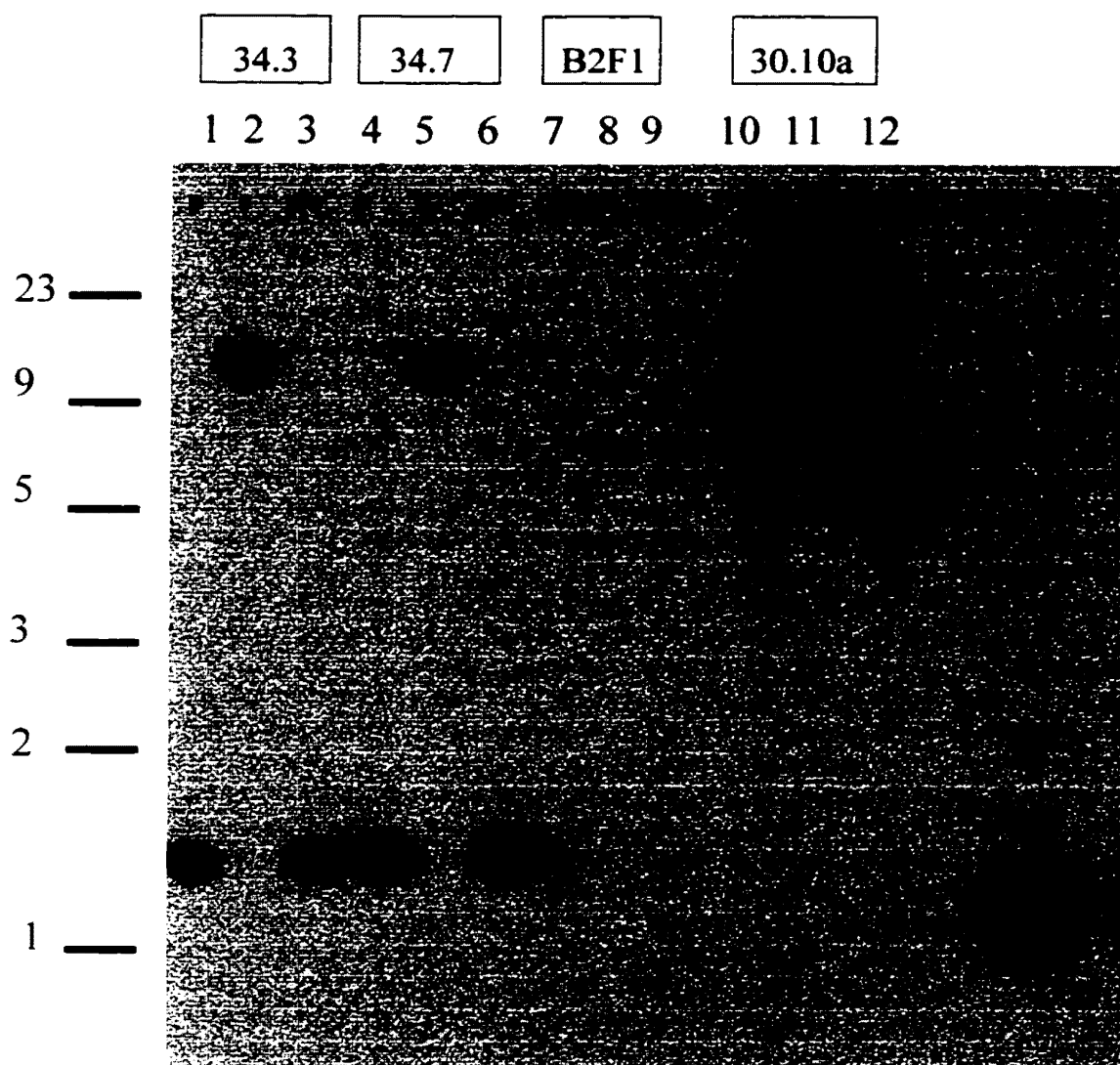


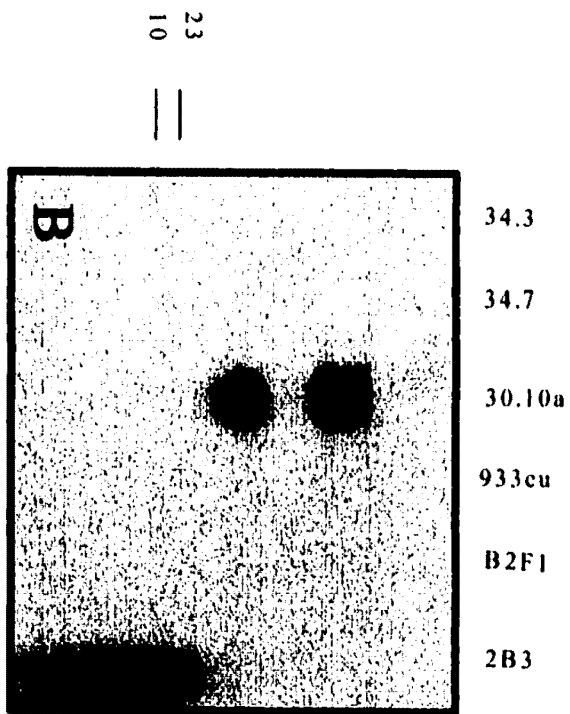
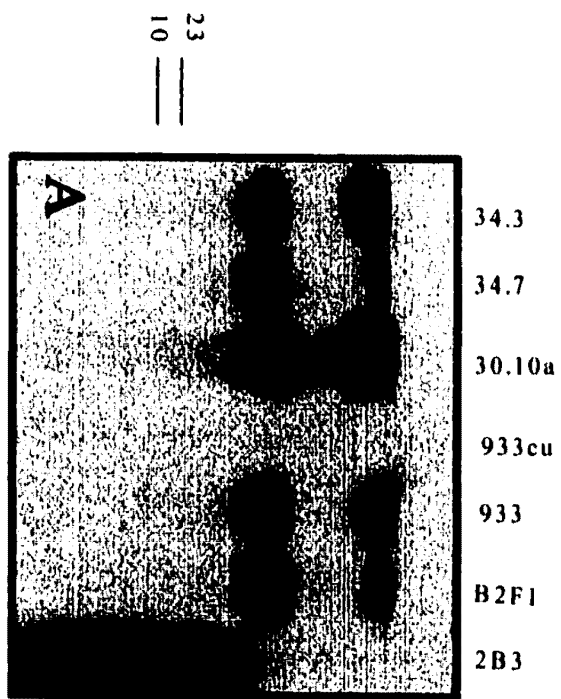
Figure 27. Southern hybridization of *Sall*-digested (lanes 2, 5, 8, and 11), *Pst*I-digested (lanes 1, 4, 7, and 10) or both *Sall* and *Pst*I-digested (lanes 3, 6, 9, and 12) total DNA from B2F1 mini-Tn5*phoA*Cm^r adherence-defective mutants 34.3 (lanes 1-3), 34.7 (lanes 4-6) and 30.10a (lanes 10-12); and the parental strain B2F1 (lanes 7-9). The probe used in the analysis was the ECL-horseradish-peroxidase-labeled chloramphenicol resistance gene from mini-Tn5*phoA*Cm^r DNA size markers (kb) are indicated.



Southern hybridization analysis of the large plasmid encoded by the adherence mutants for the presence of the mini-Tn5*phoA*Cm^r insertion. The ~90 kb plasmid that is present in B2F1 and 77-81% of non-0157:H7 STEC (Levine, et al., 1987) was purified from the parental wild-type B2F1 and mini-Tn5*phoA*Cm^r mutants, 2B3, 34.3, 34.7 and 30.10a. EHEC 0157:H7 strain 933 was used as a positive control and the plasmid-cured derivative of 933, 933cu, served as a negative control. The plasmids were isolated by the Kado and Liu method (Kado and Liu, 1981) and were virtually free of chromosomal DNA. The plasmid isolated from each strain was electrophoretically separated in a 0.6% agarose gel and transferred to a nitrocellulose membrane for Southern hybridization analysis. The DNA transfers described above were probed with CVD419 (Levine, et al., 1987), to prove that plasmid DNA had been isolated from each of the *E.coli* strains. The nicked (higher band) and super-coiled (lower band) forms of the large plasmid were detected by the probe. Plasmid preps from each of the STEC strains that carried the ~90 kb plasmid hybridized with probe CVD419 (Figure 28 panel A, detected as two bands). DNA isolated from 2B3 was denatured during processing and ran as a smear. As expected, the plasmid-cured derivative 933cu did not react with CVD419.

The same membrane was then re-probed (after the plasmid-specific probe was removed) with the Cm^r gene from mini-Tn5*phoA*Cm^r to determine if the transposon had inserted into the large plasmid encoded by the mutants (Figure 28 panel B). The ~90 kb plasmids isolated from mutants 30.10a and 2B3 both contain a mini-Tn5*phoA*Cm^r insertion and hybridized with the

Figure 28. Southern hybridization analysis of unrestricted plasmid DNA isolated from mini-Tn5*phoA*Cm^r adherence-defective strains 34.3, 34.7, 30.10a, and controls B2F1, 933cu, 933, and 2B3. The probe used in the analysis in panel A was the ECL-horseradish-peroxidase-labeled 3.4 kb *Hind*III restriction fragment from plasmid p0157 called CVD419. The probe used in the analysis in panel B was the ECL-horseradish-peroxidase-labeled chloramphenicol resistance gene from mini-Tn5*phoA*Cm^r. Position of molecular weights size markers (kb) are shown.



Cm^r gene probe. Hence, the mini-Tnp5*hoACm^r* had inserted onto the large plasmid in strain 30.10a but inserted into the chromosome of mutants 34.3 and 34.7 (Figure 28 panel B).

VII. Strategy used to clone the mini-Tn5*phoACm^r* disrupted gene from strain 34.7.

The main goal of this investigation was to identify factor(s) important for the adherence of intimin-negative STEC to human epithelial cells. To achieve this goal, an adherence mutant was identified and the gene that contained the mini-Tn5*phoACm^r* was cloned and characterized. The strategy used to clone the gene, and identify the cloned gene is discussed below.

Three mutants were isolated by transposon mutagenesis that were unable to bind to T84 cells in the same manner as wild-type. In strain 30.10a, the transposon had inserted within a gene encoded by the large plasmid. Because the plasmid-cured derivative of B2F1, called S11, adhered to T84 cells with a wild-type phenotype, this indicated that the plasmid did not encode adhesins or regulators needed for adherence to human colonic epithelial cells (T84 cells). Therefore, mutant 30.10a was not chosen for further study. Strains 34.3 and 34.7 were shown to contain the transposon insertion within the same region of DNA and were presumed to contain the same insertion. Mutant 34.7 did not appear to damage T84 cells during a standard 3 hour adherence assay and was selected as the candidate from which to clone and identify the disrupted gene.

The gene was cloned directly from the genomic DNA of 34.7 (Figure 29). *HindIII* digestion of total DNA resulted in fragments that were 6 kb in size and that hybridized to the transposon encoded Cm^r probe. The *HindIII* enzyme cut within the mini-Tn5*phoACm^r* coding

Figure 29. Strategy used to clone the gene that contained the mini-Tn5*phoA*Cm^r insertion from the adherence-deficient mutant 34.7.

Strategy used to clone from the adherence-deficient mutant 34.7 the gene that contained the mini-Tn5*phoA*Cm^r insertion



DNA was isolated from 34.7 and restricted with *Hind*III.



A 6 kb DNA fragment that contained the mini-Tn5*phoA*Cm^r insertion was identified that hybridized to the Cm^r gene.



Agarose gel slices that corresponded to 5 kb, 6 kb, and 7 kb DNA fragments, were eluted from the gel and kept as separate pools. This constituted the *Hind*III DNA library.



The *Hind*III DNA restriction library was ligated into *Hind*III digested moderate copy number plasmid pBR322 to generate pAOB347.



Plasmids were transformed into DH5 α , and transformants were selected on LB agar supplemented with chloramphenicol & ampicillin.



The 6 kb *Hind*III restriction fragment present in the recombinant plasmids were subjected to DNA analysis.

sequence but left the chloramphenicol gene marker intact. Because the *HindIII* fragment was 6 kb in size, we hoped that this fragment would contain some portion of the 3' end of the mini-Tn5*phoACm^r* disrupted gene. Gel slices were made above (7 kb), below (5 kb) and at the position of the 6 kb fragment to improve the chance that the fragment of DNA that contained the mini-Tn5*phoACm^r* insertion would be isolated. *HindIII*-restricted DNA fragments were separated on a 0.7% agarose gel (Figure 30 shows only the 6 kb pool used for ligation) and separate pools were made that consisted of DNA fragments in the size ranges of 5, 6 and 7 kb. A bank was constructed of the three different pools of *HindIII*-restricted DNA by ligating these fragments into the *HindIII* site of the pBR322 cloning vector. The resultant plasmids were transformed into the laboratory strain DH5 α . Recombinants that harbored the plasmid and that contained the 6 kb fragment of interest were selected on LB agar with chloramphenicol and ampicillin added. Two chloramphenicol and ampicillin resistant colonies were recovered and both plasmids isolated from these transformants designated pAOB347.1 and pAOB347.2, contained a 6 kb insert of the predicted size (Figure 31 is a diagram of pAOB347.1).

VIII. Identification of the gene that contained the transposon insertion.

To determine the sequence of a portion of the DNA adjacent to the mini-Tn5*phoACm^r* insertion present on the 6 kb fragment that was cloned from 34.7 onto the recombinant plasmids pAOB347.1 and pAOB347.2, primer AM11 was used that hybridized to transposon DNA located at the distal 3' end of mini-Tn5*phoACm^r* (Figure 32). Automated sequence analysis revealed approximately 300 bp of DNA encoded by mutant 34.7 that was located at the fusion

Figure 30. Agarose gel electrophoresis of the 6 kb *HindIII* DNA fragment pool derived from mutant 34.7. In panel A lane 4 contains the 6 kb fragment that was used to generate the recombinant plasmid pAOB347. Panel B (lanes 4 and 5) are the *HindIII* digested recombinant plasmid pAOB347.1 and pAOB347.2, respectively, that contain the 6 kb fragment isolated from the DNA of 34.7. Lane 3 panel B contains spillover from lane 4 i.e. pAOB347.1. Lanes marked as (M) in both panels A and B contain DNA molecular weight markers (kb).

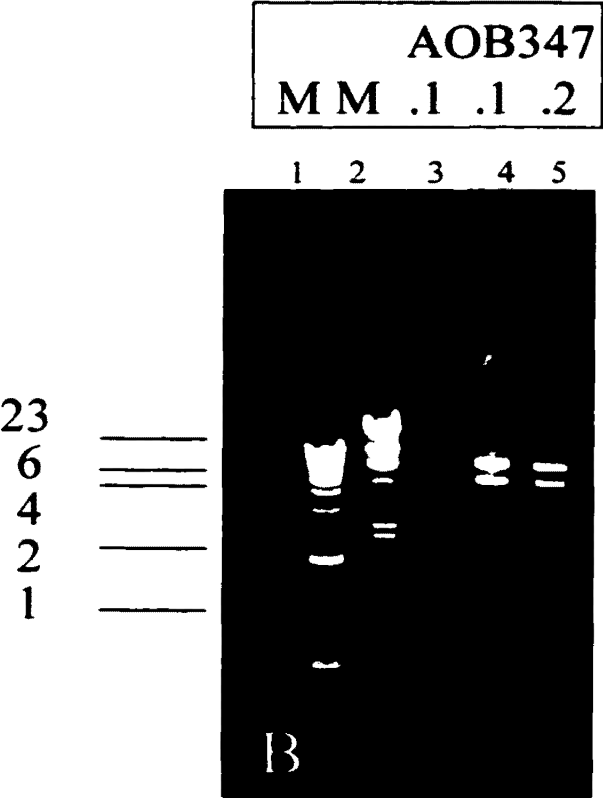


Figure 31. Drawing of plasmid pAOB347 that carries the 6 kb *HindIII* DNA fragment cloned from the mini-Tn5*phoACm*^r adherence mutant 34.7. The 6 kb fragment is represented by the grey region that encodes the chloramphenicol resistance gene derived from the mini-Tn5*phoACm*^r, and the black region that represents the gene adjacent to the 3' end of the chloramphenicol resistance gene, and the hatched portion that symbolizes the rest of the DNA cloned from mutant 34.7 at the 3' end of the chloramphenicol resistance gene. The 6 kb *HindIII* DNA fragment was ligated into the *HindIII*-digested moderate copy number cloning vector pBR322 that encodes tetracycline and ampicillin resistance. Map drawn to scale with CloneMap program version 2.11 from CGC Scientific, inc.

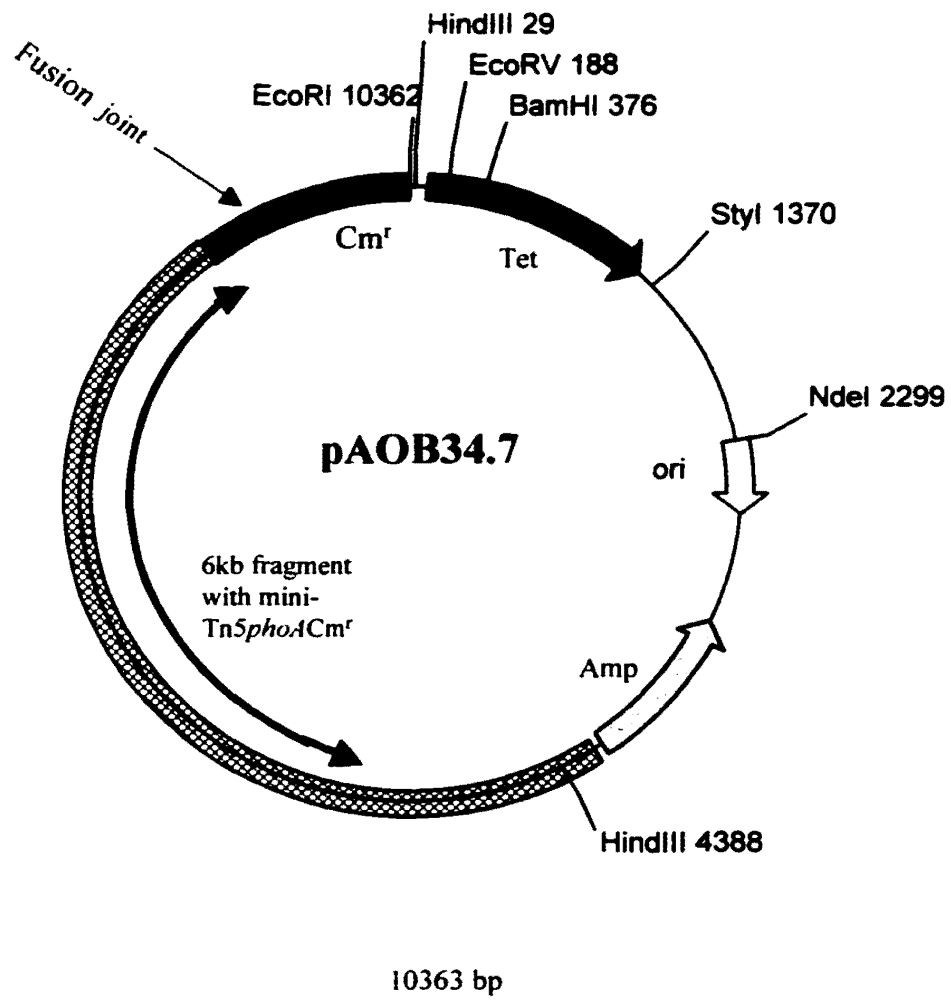
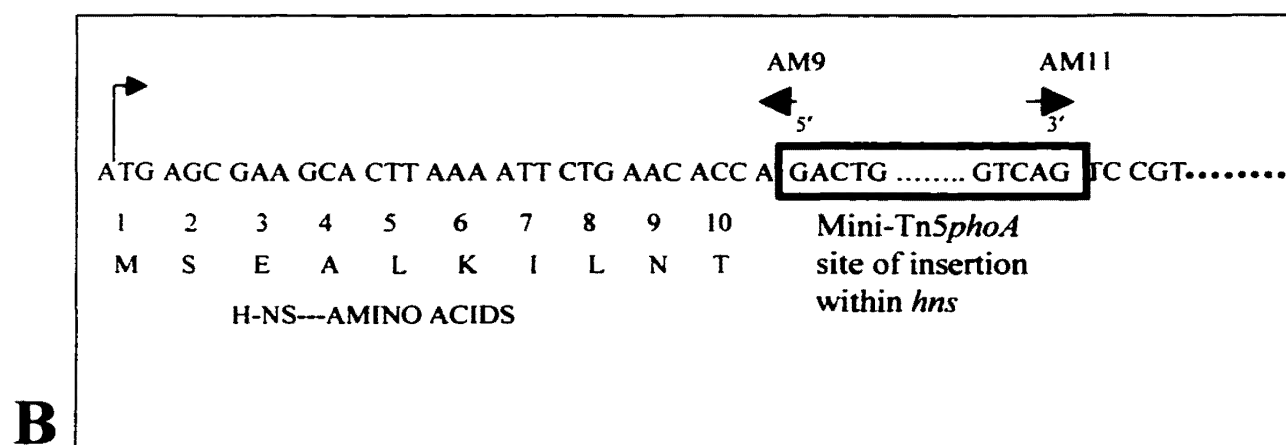
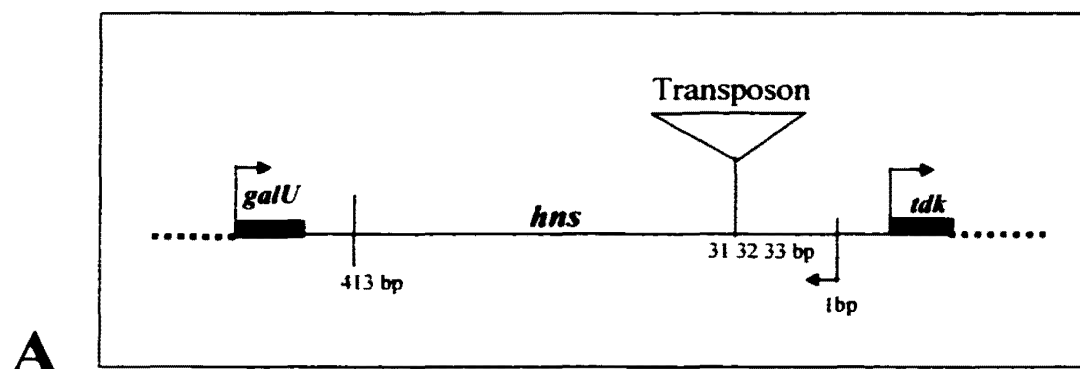


Figure 32. Location of mini-Tn5*phoA*Cm^r within the *hns* gene encoded by adherence-deficient mutant 34.7. Panel A shows the location of the transposon within the *hns* gene open-reading-frame flanked by *galU* and *tdk*. Small arrows indicate direction of gene transcription. Panel B indicates the site of insertion of mini-Tn5*phoA*Cm^r within *hns*. The transposon is represented by an inverted repeat of GACTG.....GTCAG enclosed in a box located within the 11th codon of the *hns* nucleotide sequence. Primer set AM9 and AM11 (large black arrowheads in panel B) was used to determine the DNA sequence at the 5' and 3' ends of the mini-Tn5*phoA*Cm^r-gene fusion joints. This Figure is not drawn to scale.



joint between mini-Tn5*phoACm*^r and the gene into which the transposon had inserted. The Basic Local Alignment Search Tool (BLAST) was used to match the 300 bp of DNA cloned from 34.7 to relevant gene sequences present in the nucleic acid database maintained by the National Center for Biotechnology Information. The 300 bp of DNA derived from mutant 34.7 matched the *E. coli hns* gene.

IX. The site of insertion of the transposon within the *hns* gene.

Sequencing of the 6 kb fragment at the 3' end of the mini-Tn5*phoACm*^r gene fusion in *hns* indicated that the insertion occurred out-of-frame between the first and the second nucleotide of the eleventh codon (isoleucine) of *hns* (Figure 32). Because the out-of-frame insertion of mini-Tn5*phoACm*^r occurred early in the *hns* sequence neither a functional H-NS nor mini-Tn5*phoACm*^r phosphatase protein could have been made.

Because, mutant 34.7 produced blue colonies on XP media, we had initially assumed that the mutant contained an in-frame insertion within a gene that encoded an exported or surface exposed protein. We now speculated that the absence of H-NS in 34.7 may have de-repressed the transcription of alkaline phosphatase and this could have caused the blue colonies detected when 34.7 was grown on XP media. Other investigators have reported similar findings when H-NS is inactivated (Bertin et al., 1994; Harrison et al., 1994 and Woolwine et al., 1998).

X. Sequence analysis of *hns* encoded by 34.7, an *hns* knockout and adherence-deficient mutant of B2F1.

Primer AM9, transposon specific primer, was used to obtain the sequence information at the 5' end of the *hns*::mini-Tn5*phoACm*^r fusion joint (Figure 32). The complete *hns*

sequence derived from 34.7 was then aligned with the *hns* encoded by K-12, a non-pathogenic *E.coli*, with the aid of the BLAST program. The Kohara cosmid clone (clone #250, accession: D90759) was used for the alignment. This clone corresponds to the 27.6 through the 28 minute region of the linkage map prepared by Oshima et al., in 1996. The results indicated that the *hns* gene encoded by B2F1 is almost identical to that encoded by *E.coli* K-12 (Figure 33). In B2F1 the tenth codon has been replaced by threonine (bolded and underlined in Figure 33); which is an asparagine in *E.coli* K-12. Furthermore, the orientation of the transcriptional unit of *hns* encoded by B2F1 was the same as in K-12 *E. coli* (Figure 32). The *hns* gene encoded by B2F1 and K-12 *E. coli* is transcribed (5' to 3') counter clockwise and is located between the *galU* and *tdk* genes. Both *galU* and *tdk* are transcribed (5' to 3') clockwise in the opposite orientation of the *hns* gene. Intervening non-coding regions of DNA separate the three genes.

XI. Isolation of the wild-type *hns* gene from B2F1.

Based on the sequence of *hns* from B2F1, primers (MS4 and MS5) were designed for (PCR) amplification of *hns* and flanking *galU* and *tdk* sequences directly from the chromosome of wild-type B2F1. Figure 34 shows the location of the primers used for PCR amplification. *Bam*HI restriction sites were engineered into the primers so that the PCR DNA product would contain this restriction site at both its 5' and 3' ends. The DNA product obtained after PCR amplification was purified by extraction from an agarose gel with the GeneClean spin system and then ligated into the cloning vector pCR2.1 (Clark, 1988 and Mead et al., 1991) to generate pAOB100, Figure 35. The pCR2.1 cloning vector contains 3'-T overhangs for direct ligation of *Taq* amplified PCR products. The recombinant plasmids were transformed into DH5 α , and

Figure 33. Nucleic acid sequence of *hns* encoded by STEC 091:H21 strain B2F1.

Nucleic acid sequence of *hns* encoded by

STEC 091:H21 strain B2F1

```
1  ATGAGCGAAACACTTAAAATTCTGAACACCATCCGTACTCTTCGTGCGCA
51  GGCAAGAGAATGTACACTTGAAACGCTGGAAGAAATGCTGGAAAAATTAG
101 AAGTTGTTGTTAACGAACGTCGCGACGAAGAAAGCGCGGCTGCTGCTGAA
151 GTTGAAGAGCGCACTCGTAAACTGCAGCAATATCGCGAAATGCTGATCGC
201 TGACGGTATTGACCCGAACGAACCTGCTGAATAGCCTTGCTGCCGTAAAT
251 CTGGCACCAAAGCTAAACGTGCTCAGCGTCCGGCAAAATATAGCTACGTT
301 GACGAAAACGGCGAAACTAAAACCTGGACTGGCCAAGGCCGTACTCCAGC
351 TGTTATCAAAAAAGCAATGGATGAGCAAGGTAAATCCCTCGACGATTTC
401 TGATCCAGCAATA
```

Figure 34. Location of the primers used to amplify and to sequence the 1882 bp DNA PCR product from the chromosome of wild-type strain B2F1. The large grey arrow shows the location of *hns* and direction of transcription of the gene. The small black arrows at the distal 5' and 3' ends are the location of primers MS4 and MS5 that were used to amplify the 1.9 kb DNA product from the chromosome of B2F1. Location of the *Bam*HI (B) sites engineered onto the ends of the PCR fragment are indicated. The primers used to sequence the 1.9 kb region overlapped each other (large black arrows with locations of the start of each primer is designated in base pairs) and hybridized to sequences on the top-strand (5'–3') of the 1.9 kb PCR product. The location of the start sites of *galU* and *tdk* are shown. Drawn to scale with the CloneMap program.

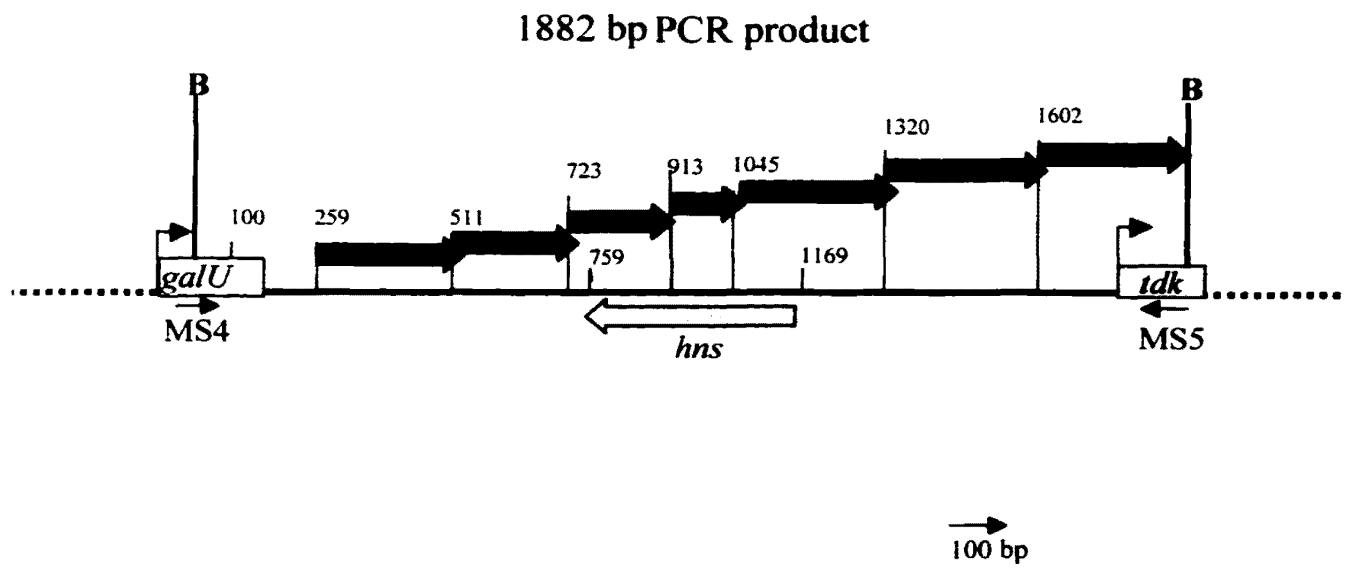
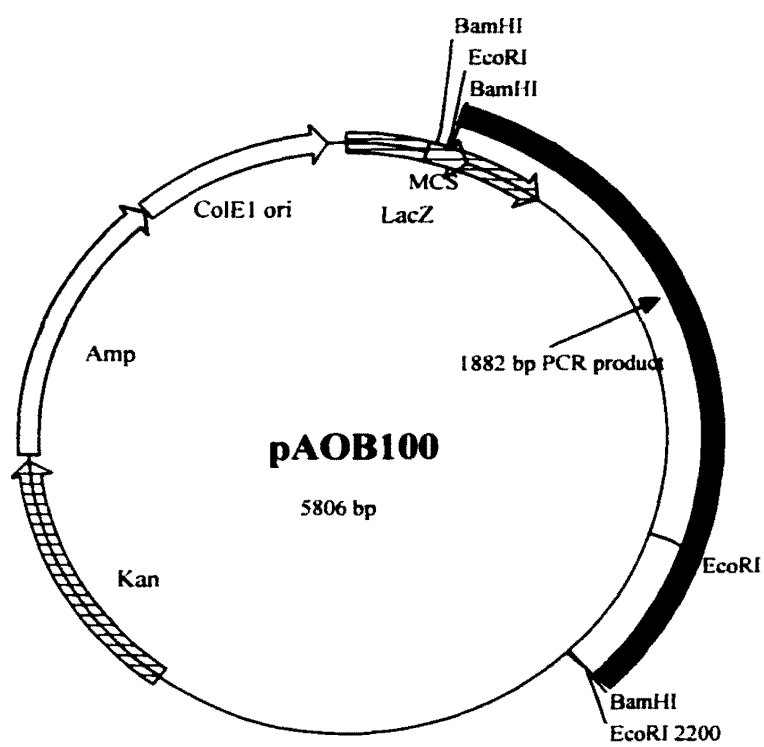


Figure 35. Diagram of plasmid pAOB100 that carries the 1882 bp region of DNA cloned from the chromosome of wild-type B2F1. The 1.9 kb region encodes *hns* and flanking *galU* and *tdk* gene sequences. Primer set used for PCR amplification was designated as MS4 and MS5 (position of primers are shown in Figure 32). The 1.9 kb PCR product (black region) was ligated into the multiple cloning site of vector pCR2.1 to generate pAOB100. This plasmid confers kanamycin (hatched) and ampicillin (grey) resistance.



transformants were selected on media that contained ampicillin. Purified plasmid was isolated from one of the selected transformants, and the 1.9 kb insert was sequenced. The location of the primers that were used to sequence the insert that contained *hns* and a portion of *galU* and *tdk* are shown in Figure 34.

XII. Analysis of Shiga-toxin and STEC hemolysin production in the *hns* null mutant 34.7 deficient in adherence to colonic epithelial T84 cells.

Shiga toxin production by *hns* mutant 34.7. The relevance of H-NS as a regulator of the expression of Shiga toxin in STEC has not been investigated. Because inactivation of H-NS has been shown to affect the expression of other virulence genes encoded by various strains of pathogenic *E. coli* (Colonna et al., 1995 and White- Ziegler et al., 1998); we were interested in whether inactivation of *hns* in 34.7 had any effect on the expression of Stx2d. Although T84 cells are not sensitive to Stx2d production by B2F1, there was a possibility that if the levels of Stx expressed by mutant 34.7 were increased, T84 cells could be damaged enough to prevent adherence of 34.7 to them. For this reason, the levels of Shiga toxin produced by the *hns* mutant 34.7 was compared to the levels of toxin produced by wild-type B2F1. Because H-NS has been shown, in some cases, to exert its effect at 30°C on genes that encode virulence factors, Stx2d production by B2F1 and 34.7 were compared at 30°C and 37°C (Figures 36 and 37, respectively). The Vero cell cytotoxic assay was used to determine the level of toxin produced by these bacteria.

Figure 36 Shiga toxin levels produced by the *hns::mini-Tn5phoACm^r* adherence mutant strain, 34.7 compared to the levels of Shiga toxin produced by the wild-type strain, B2F1 at 30°C. Filtrates of sonic extracts of stationery phase cultures were prepared and tested on Vero cells to determine the cytotoxicity of each preparation. The cytotoxic titer per milliliter of culture was expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a given well (CD₅₀/ml). The geometric mean of three separate tests are shown. Not shown, lysates of non-toxin-producing negative control strain, DH5α, were less than 10¹ CD50/ml.

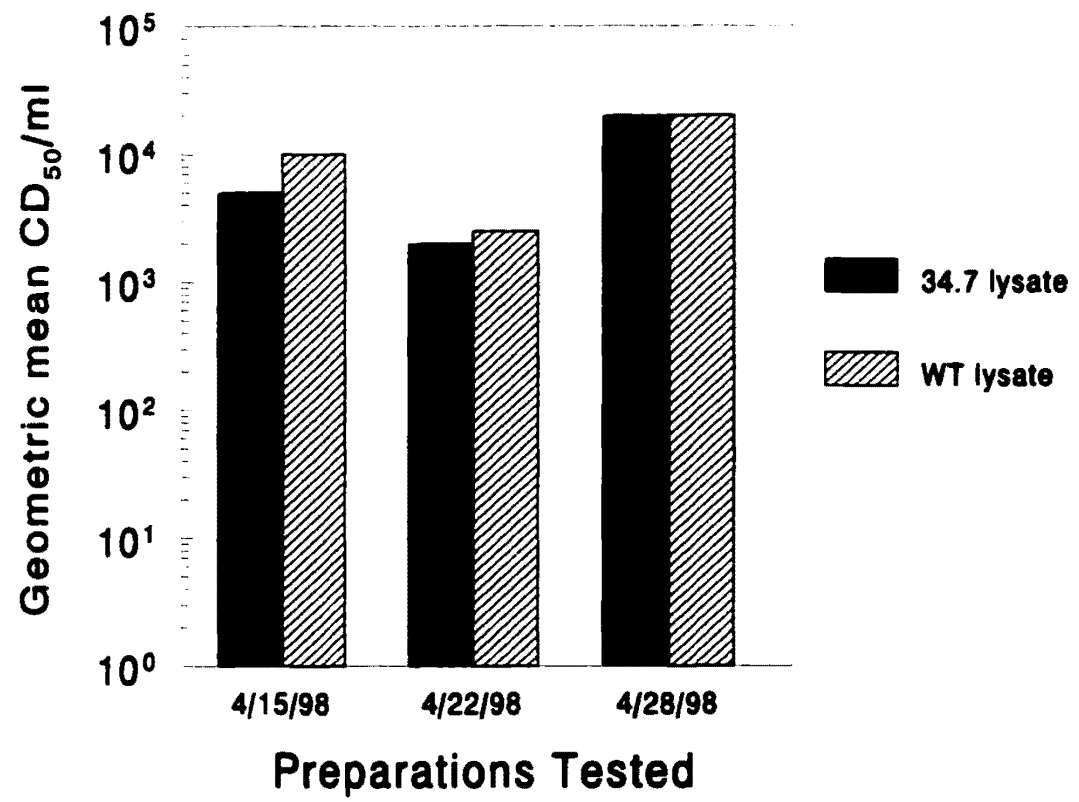
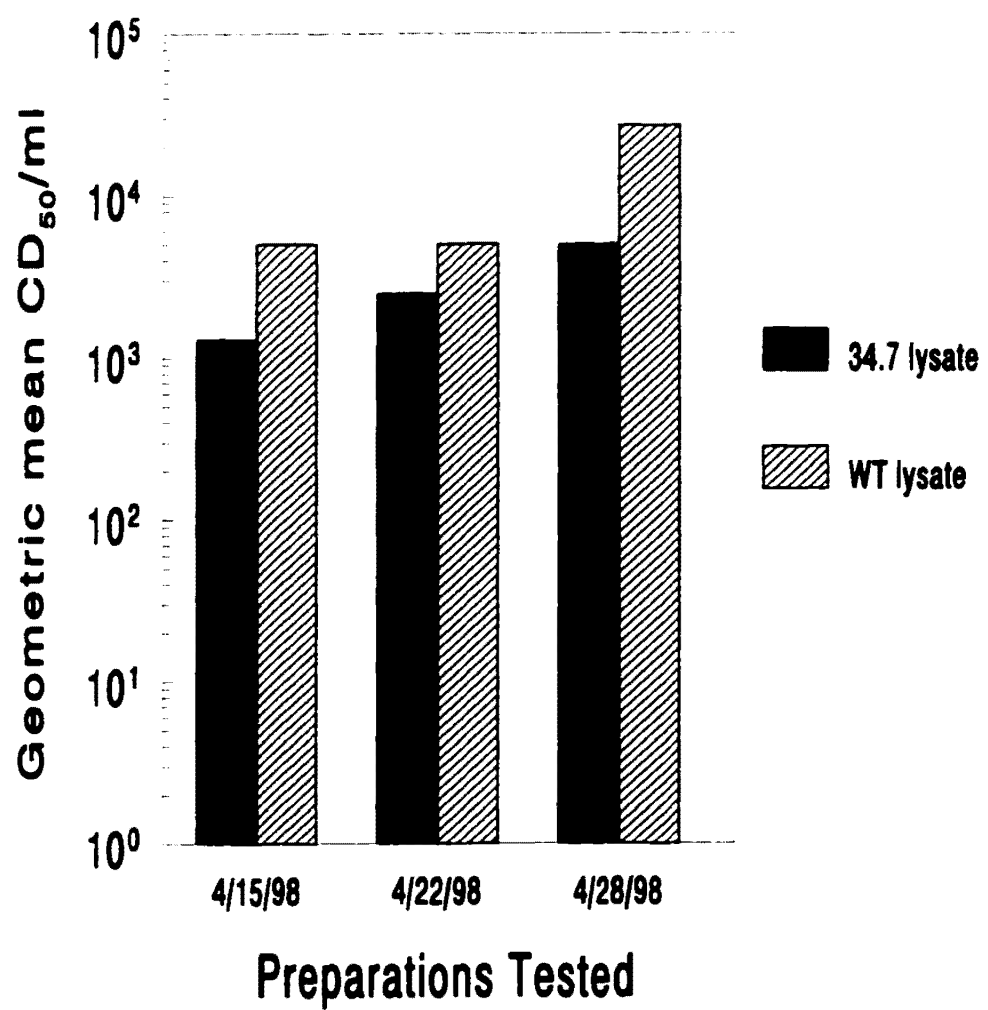


Figure 37. Shiga toxin levels produced by the *hns::mini-Tn5phoACm^r* adherence mutant 34.7 compared to the levels of Shiga toxin produced by the wild-type strain, B2F1 at 37°C. Filtrates of sonic extracts of stationery phase cultures were prepared and tested on Vero cells to determine the cytotoxicity of each preparation. The cytotoxic titer per milliliter of culture was expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a given well (CD₅₀/ml). The geometric mean of three separate tests are shown. Not shown, lysates of non-toxin-producing negative control strain, DH5α, were less than 10¹ CD50/ml.



Shiga toxin is a periplasmic protein that is produced during logarithmic phase of growth, and growth at 37°C is optimal for toxin production (reviewed by O'Brien et al., 1992). Hence, B2F1 and 34.7 were grown to stationary phase at 30°C and 37°C for comparison of Stx2d production. Filtrates of the sonic extracts were tested on Vero cells to determine the cytotoxicity. The cytotoxic titer per milliliter of culture was expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a given well (CD_{50}/ml). Three separate tests were done, in duplicate, at both temperatures, and the CD_{50}/ml (titers) were compared. Titers ranged from greater than 10^3 CD_{50}/ml to approximately 10^5 CD_{50}/ml and there were no pronounced differences in the amounts of Stx2d produced by B2F1 and 34.7. Nor did the temperature at which the bacteria were grown appear to be a factor in the levels of toxin produced (see Figures 36 and 37). In conclusion, *hns* does not appear to have a role in the regulation of Stx2d toxin production by STEC strain B2F1. Hence, the reduced adherence of 34.7 can not be explained by increased levels of Stx2d interfering with bacterial-host cell interaction.

Titer of STEC-hemolysin secreted by the *hns* mutant and wild-type

B2F1. Strain 34.7, a *hns* null mutant of B2F1, produced large zones of hemolysis on agar that contained washed SRBCs and these zones were in contrast to wild-type B2F1 that produced small turbid zones of hemolysis. Typically hemolytic activity was not detected in the cell free filtrates of wild-type B2F1 or other STEC (Schmidt et al., 1995). Taken together, these results suggested that the mutant 34.7 might secrete hemolysin and this hemolysin might reduce adherence to T84 cells. As a first step to test this theory, the secreted hemolytic activity of 34.7 was quantified. A hemolysis tube assay was used based on the method of Welch et al. (Welch

et al., 1983). Briefly, five milliliter cultures at an OD_{600} of 1.0 were centrifuged, the supernatants were harvested and filtered. The hemolytic titer was determined and expressed as the reciprocal of the dilution where the lysis of the erythrocytes was evident after two hours incubation at 37°C. Figure 38 shows the OD_{540} reading obtained from undiluted samples. This representative assay indicated that strain 34.7 secreted hemolysin (the titer was 8), whereas, the wild-type strain had no detectable hemolytic titer by tube assay. Mutant 34.3 also secreted hemolysin (the titer was 4). The negative control, S11 (the plasmid-cured derivative of B2F1) secreted no detectable hemolysin. These results indicate that the *hns* adherence mutant 34.7 secretes an abnormal amount of hemolysin.

Comparison of the level of STEC hemolysin produced by 34.7 and wild-type B2F1 by SDS-PAGE and Western blot analysis. Protein profiles of 34.7 and B2F1 were compared by SDS-PAGE electrophoresis to determine the relative amounts of hemolysin produced by each strain; the molecular weight of STEC hemolysin corresponded to a band of 107-kDa. The samples for the SDS-PAGE gel included the supernatant (S), the pellet (P) and the total lysate with culture supernatant (T) from strains B2F1, S11 and 34.7. In panel A of Figure 39 and in the lanes marked as 34.7 a heavier band at 107-kDa (denoted by an arrowhead) was present when compared to lanes that contain B2F1 samples (Figure 39). S11, the plasmid-cured derivative of B2F1, does not produce STEC hemolysin and served as the negative control. The band corresponding to the size of STEC hemolysin from 34.7 was primarily found in the supernatant. Note that protein present in these samples were trichloroacetic acid precipitated prior to SDS-PAGE analysis.

Figure 38. Comparison of the extracellular STEC-hemolysin activity detected in the culture supernatants of adherence mutant 34.7, 34.3, B2F1, DH5 α (hemolysin negative) and S11 (hemolysin negative). A representative assay is shown and only undiluted samples were graphed for comparison. The maximum lysis (SRBCs plus water) was 1.5 OD₅₄₀ and the blank (LB broth plus SRBCs) was 0.04 OD₅₄₀.

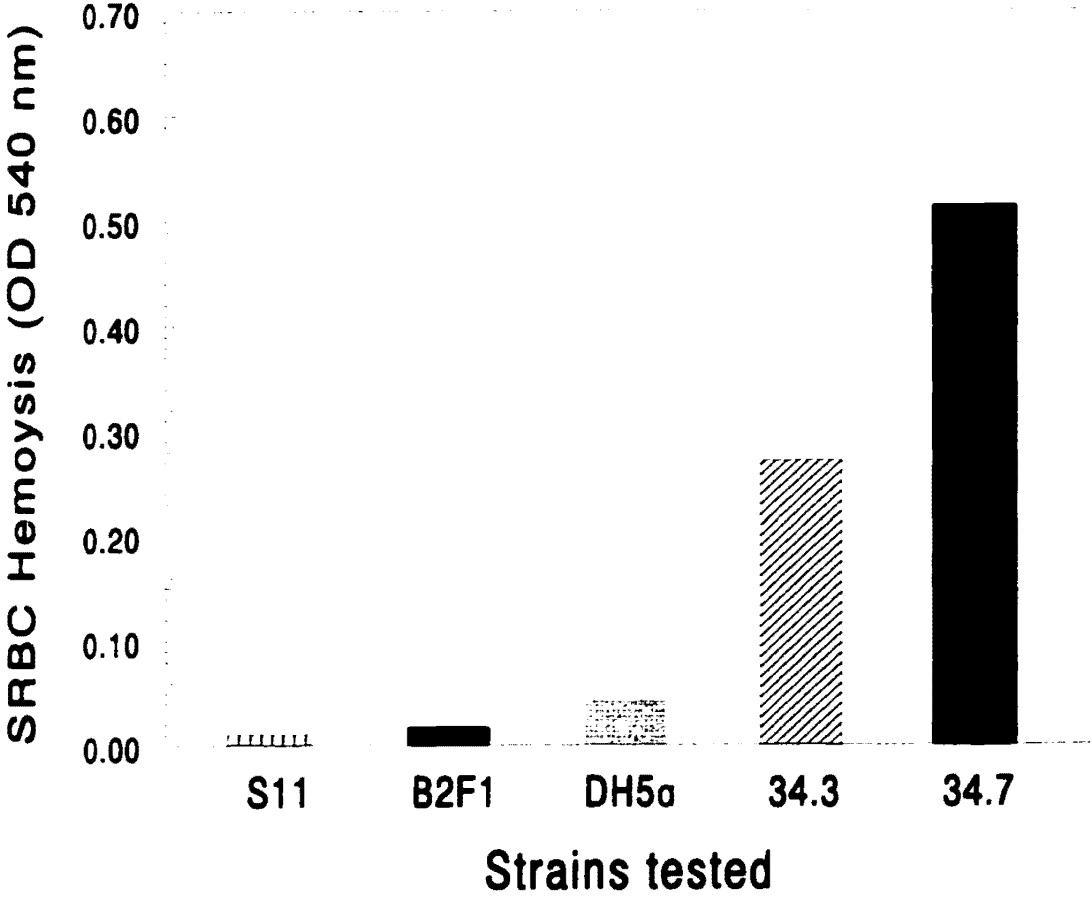
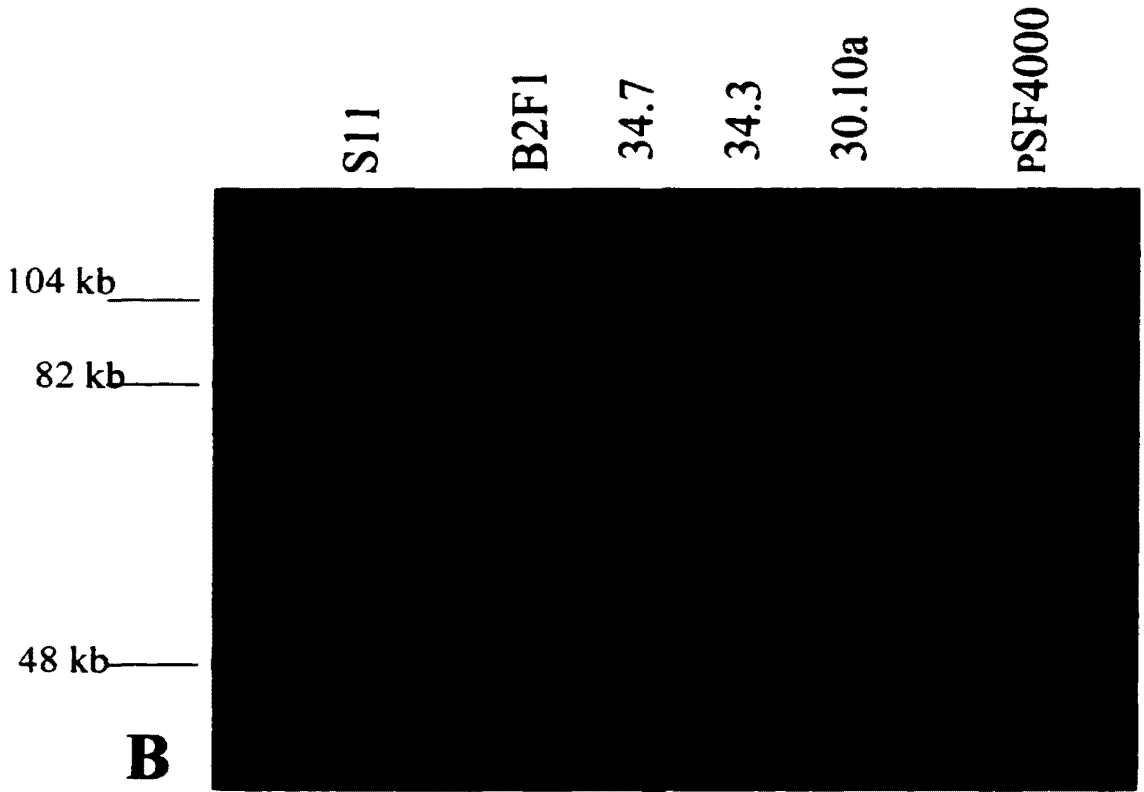
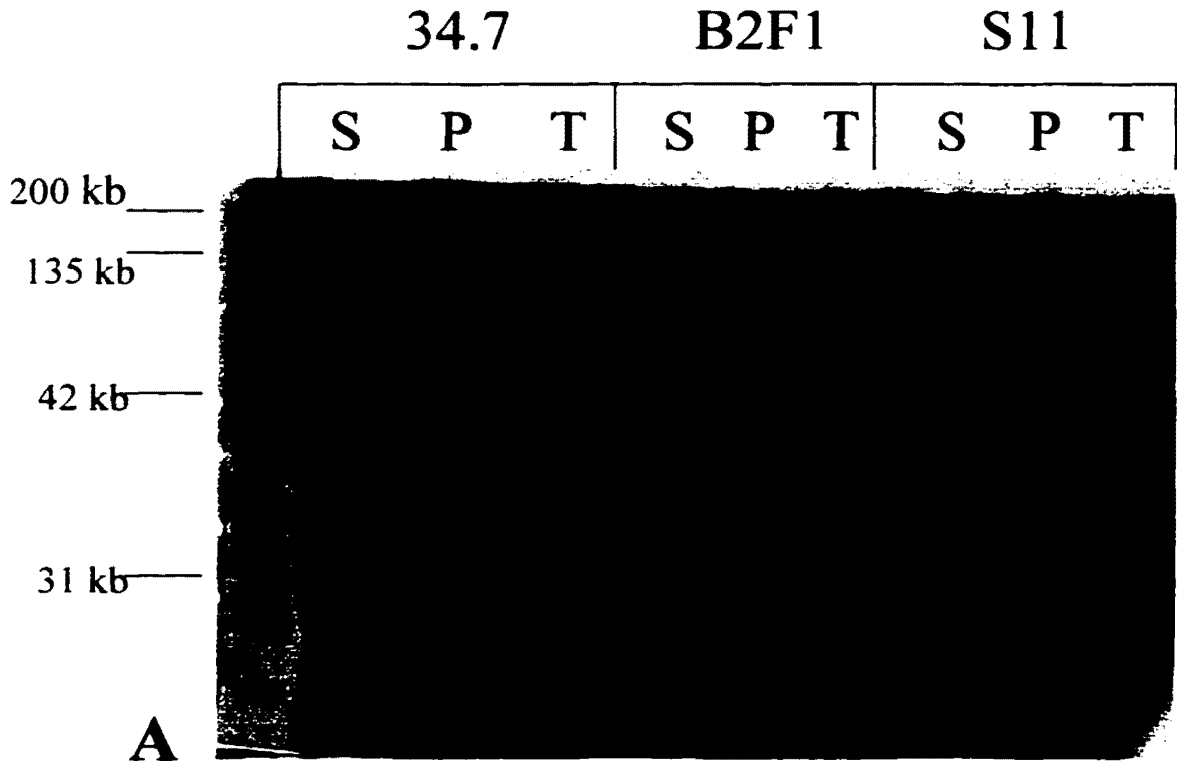


Figure 39. Comparison of the amount of STEC hemolysin protein produced by adherence mutant 34.7, 34.3 and wild-type, B2F1 by SDS-PAGE (panel A) and Western blot analysis (panel B).

Panel A represents the protein profiles of 34.7, B2F1, and S11 present in the culture supernatant (S), pellet (P) and total sonic extract (T) fractions of stationary phase cultures. The proteins in each fraction reflect equivalent colony forming units per milliliter. The position of the 107-kDa band that represents the STEC hemolysin is marked by a small black dot in the supernatant fractions for both B2F1 and 34.7.

Panel B is a Western blot done on a different day (samples in this gel do not correspond to the samples shown above). Sonic extracts (includes the culture supernatant) of 34.3 and 34.7, 30.10a, B2F1 and S11 were prepared at the same time from cultures that had reached OD₆₀₀ of 1.0. Strain PSF4000 carries a low copy number plasmid that encodes the α -hemolysin against which the anti-serum used in the assay was prepared. The position of the 107-kDa band present in 34.3 and 34.7 samples is marked by a small black arrowhead. Position of molecular weight markers (kb) are labeled in both panels.

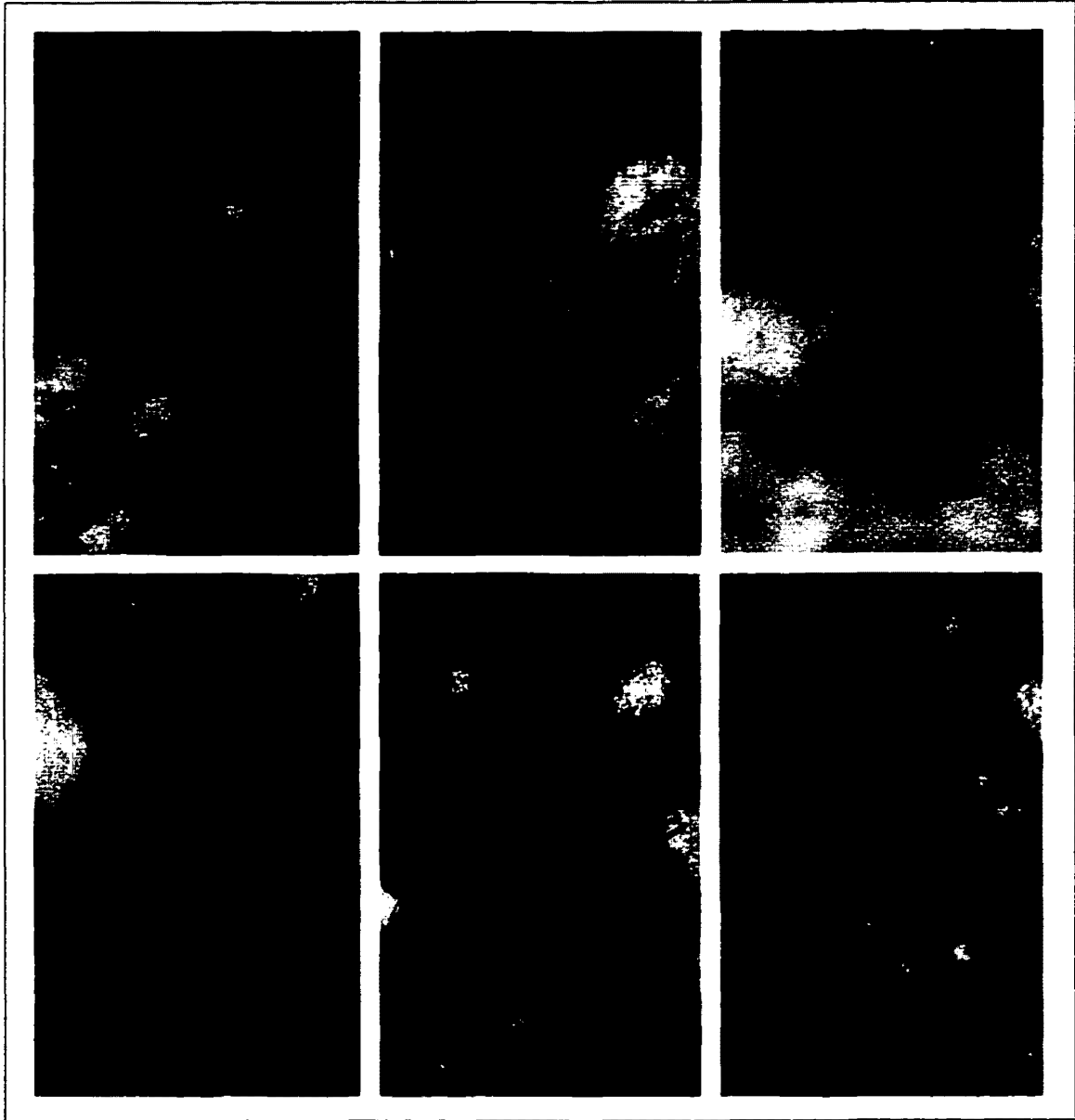


That this 107-kDa band was in fact STEC hemolysin was demonstrated with a polyclonal antiserum prepared against *E. coli* α -hemolysin as the probe. Fortunately, this anti- α -hemolysin serum (gift from Dr. Rodney Welch, Dept. Medical Microbiology, University of Wisconsin) had been previously shown by Welch and colleagues (personal communication) to cross-react with STEC hemolysin. The 107-kDa hemolysin band of protein was apparent in samples 34.7, 34.3 but not in B2F1, S11 or mutant 30.10a samples (Figure 39 panel B). In sample pSF4000, the 110-kDa α -hemolysin band was present [pSF4000 is an α -hemolysin expressing clone in *E. coli* WAF100 (Bauer et al., 1996)]. Adherence mutants deficient in H-NS, 34.7, 34.3, and the reference strain WAF100 (pSF4000) produce greater amounts of hemolysin than was produced by wild-type B2F1 (protein samples were not trichloroacetic acid precipitated). Because the proteins of the whole cell extracts were not precipitated before use, there probably wasn't a sufficient amount of STEC hemolysin in the B2F1 sample to be detected by the cross-reactive α -hemolysin antibody. In conclusion, the SDS-PAGE, and Western blot analysis, taken with the hemolytic tube assay results, indicated that STEC hemolysin production was deregulated in the *hns* mutant, 34.7, and in mutant 34.3.

XIII. H-NS regulates attachment of STEC 091:H21 to T84 cells and hemolysin production.

When *hns*, carried on a moderate copy number plasmid, pTHK113, (kindly provided by Dr. Thomas Kawula, at the University of North Carolina) was introduced into mutant 34.7 (shown in panel B of Figure 40) the adherence phenotype was restored to that of wild-type (Figure 40 panel F). The 34.7 mutant transformed with the pBR322 vector was not adherent

Figure 40. Restoration of the adherence phenotype of mutant 34.7 to that of the parent isolate B2F1 in the presence of wild-type H-NS. Mutant 34.7 was transformed with plasmid pTHK113 (moderate copy number plasmid) and a three hour adherence assay on polarized T84 cells was done. Assay results are presented for each strain as, panel A, B2F1; panel C, B2F1(pBR322); panel E, B2F1(THK113); and panel B, 34.7; panel D, 34.7(pBR322); panel F, 34.7(THK113). Black arrows indicate clumps of adherent bacteria.



(Figure 40 panel D), and wild-type B2F1 adherence was not adversely affected by the presence of plasmids pBR322 or pTHK113 [Figure 40 panel A, B2F1 alone; panel C, B2F1 (pBR322); and panel E, B2F1(pTHK113)]. These findings confirm that the *hns* mutation in 34.7 was responsible for the defect in adherence of that strain. Similarly, when mutant 34.7 was transformed with *hns* (carried on pTHK113) the hemolytic wild-type phenotype was restored, i.e. small turbid zones of hemolysis on SRBC agar (data not shown). Therefore, the presence of H-NS conferred production of wild-type levels of hemolysin on mutant 34.7.

XIV. Construction of an *hns* mutation in the hemolysin-negative strain, S11.

We were still concerned that excess STEC hemolysin production by 34.7 may have caused damage to T84 epithelial cells that was not readily discernible during the adherence assay and that this damage could be directly responsible for the reduced adherence phenotype of 34.7. Consequently, we sought to introduce the *hns* mutation from 34.7 into S11, a plasmid-cured, and hence hemolysin-negative variant of B2F1.

For this purpose, primer set MS4 and MS5 was used to clone DNA from mutant 34.7 that included the mini-Tn5*phoAC*^{cm^r} insertion within *hns* and portions of the flanking *galU* and *tdk* genes (Figure 41). The PCR product generated was 4.3 kb in size, included all of the *hns*::mini-Tn5*phoAC*^{cm^r} and a portion of the *galU* (609 bp) and *tdk* (107 bp) genes that flanked the *hns* gene. In Figure 39 small grey arrowheads represent the primers used to amplify the 4.3 kb region of DNA from the 34.7 chromosome. The 4.3 kb amplified DNA product was ligated into the cloning vector pCR2.1 to generate pCR1211 (Figure 42). Subsequently, the 4.3 kb

Figure 41. Mini-Tn5*phoACm'* insertion within the *hns* gene encoded by adherence mutant 34.7 and the primers used to amplify the 4.3 kb fragment from its chromosome. The dashed line drawn between primers MS4 and MS5 (grey arrowheads) represent the internal fragment (4.3 kb) that was ligated into the pCR2.1 cloning vector to generate pCR1211. The double line drawn between primers MS6 and MS2 (large black arrows) indicate the predicted external PCR fragment (~4.5) obtained from mutant 34.7.

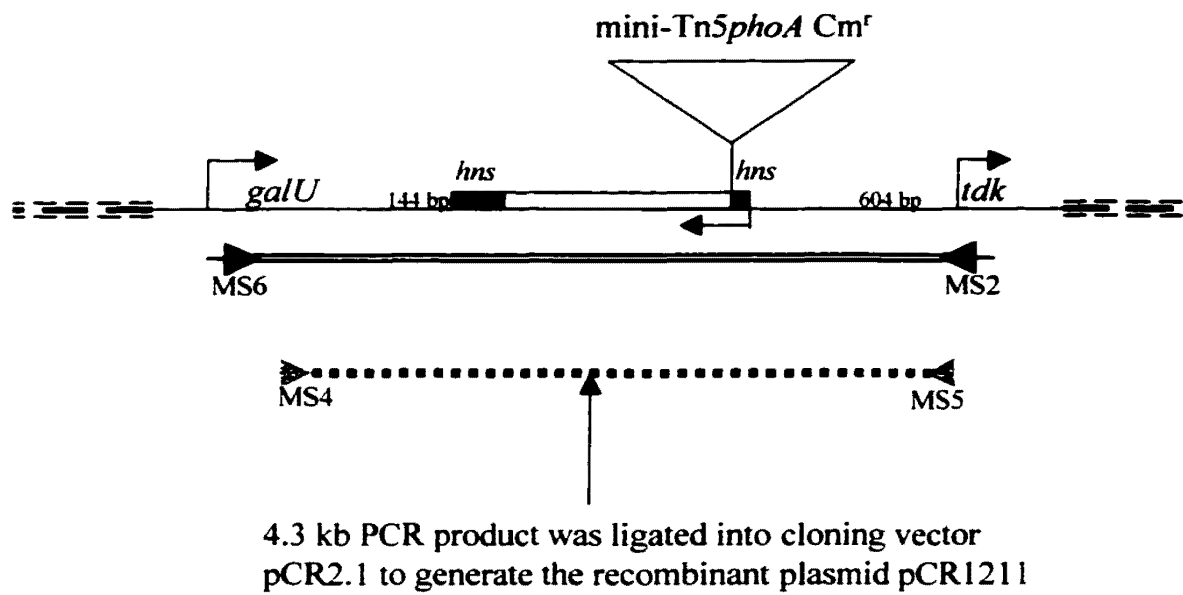
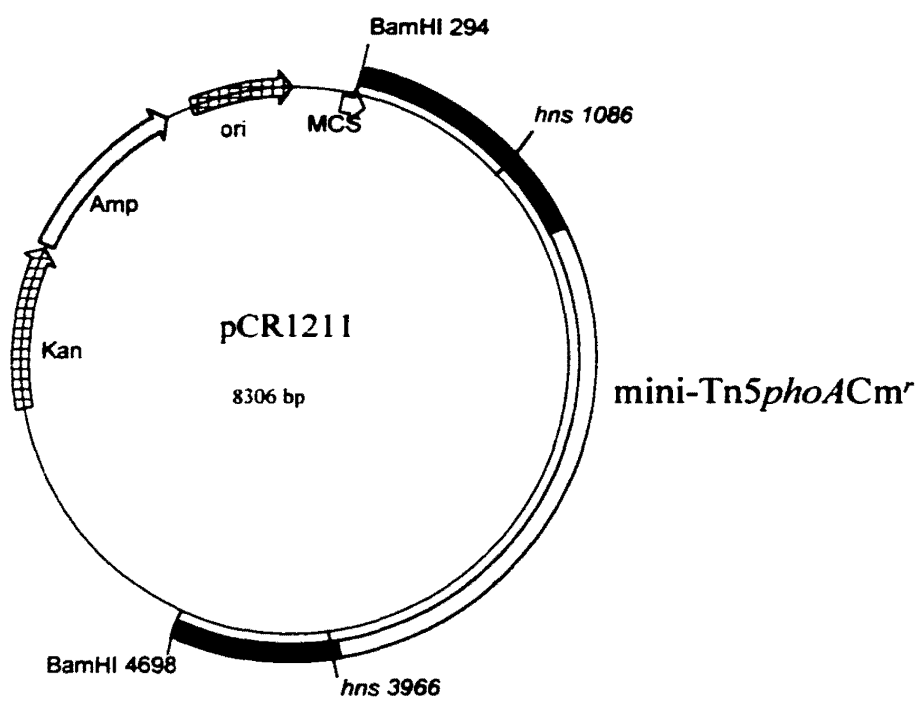


Figure 42. Plasmid pCR1211. This plasmid contains the 4.3 kb PCR fragment cloned from mutant 34.7. The 4.3 kb fragment that contained the mini-Tn5*phoAC*^r insertion within *hns* is represented on pCR1211 as a black region interrupted by a white region. The location of the disrupted *hns* gene within the 4.3 kb fragment is shown (*hns* 1086-*hns* 3966). Plasmid pCR1211 encodes kanamycin and ampicillin resistance. Map is drawn to scale.



insert was moved from pCR2.1 into the *Bam*HI site of the suicide vector pSTAMP (Melton-Celsa, this study) the recombinant plasmid was designated as pMS1211 (Figure 43). Plasmid pSTAMP, a derivative of pMAK705, (Hamilton et al., 1989) contained a temperature sensitive (ts) origin of replication, and encoded ampicillin resistance. Since plasmid pMS1211 is a derivative of pSTAMP, it also contained a ts origin of replication and as a consequence, pMS1211 replicated at 30°C but did not replicate well at 44°C. The temperature-sensitive plasmid, pMS1211, was electroporated into S11 and transformants that were ampicillin and chloramphenicol resistant were selected at 30°C. Then, stationary phase cultures of ten S11(pMS1211) transformants were diluted (10-fold), plated onto media containing chloramphenicol and ampicillin, and incubated overnight at 44 °C. Eight putative cointegrates that grew well at 44 °C on media supplemented with chloramphenicol and ampicillin were recovered. These colonies were presumed to be cointegrates that had undergone homologous recombination (single cross-over occurred between *galU* and *tdk* genes that flank *hns* on the chromosome and the partial *galU* and *tdk* that flanked *hns*::mini-Tn5*phoACm*^r on the suicide plasmid). Single cross-over homologous recombination resulted in the insertion of the whole pMS1211 plasmid into the chromosome of S11 (Figure 44 is a schematic representation of the cointegrate). The putative cointegrates were screened by PCR with primers MS6 and AM11, and four of the eight cointegrates yielded the predicted 1.4 kb DNA PCR product (data not shown). To obtain a double crossover, the four cointegrates were passaged ten times in LB broth that contained ampicillin at 30°C. Approximately one-thousand colonies were replica plated, screened on media containing ampicillin or chloramphenicol, and eight possible double

Figure 43. Suicide plasmid pMS1211. Plasmid pMS1211 was used for allelic exchange of wild-type *hns* for mutant *hns* in the plasmid-cured derivative of B2F1, designated as S11. This plasmid contains the *Bam*HI 4.3 kb fragment cloned from pCR1211 and then ligated into the *Bam*HI site of the temperature sensitive suicide plasmid pSTAMP. Plasmid pMS1211 encodes ampicillin resistance. Map is drawn to scale.

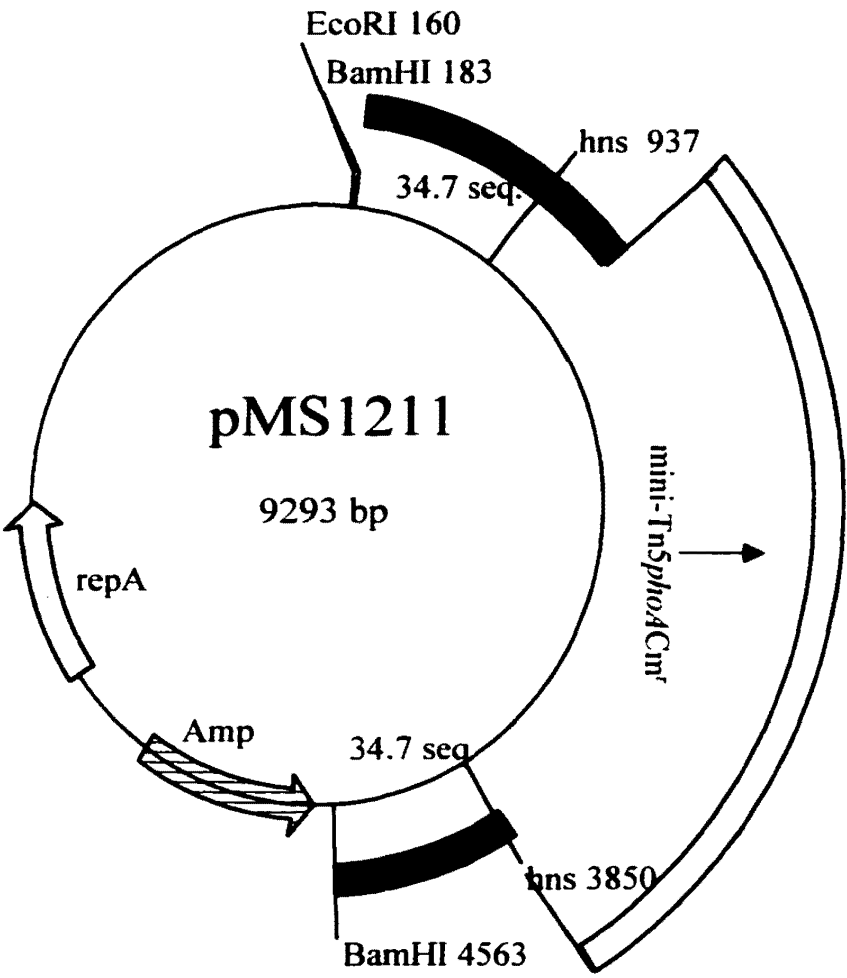
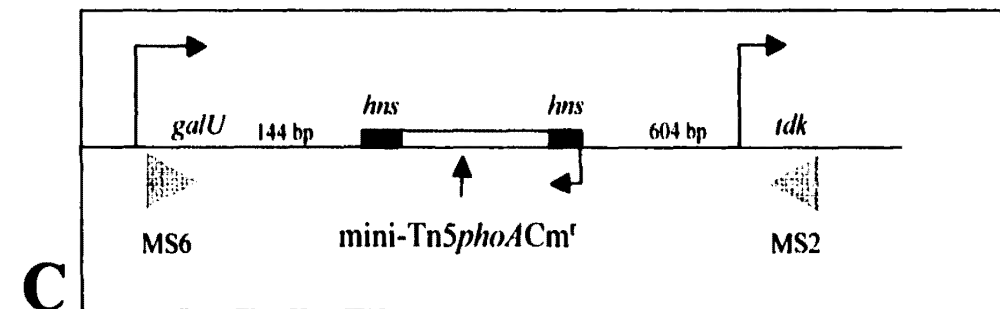
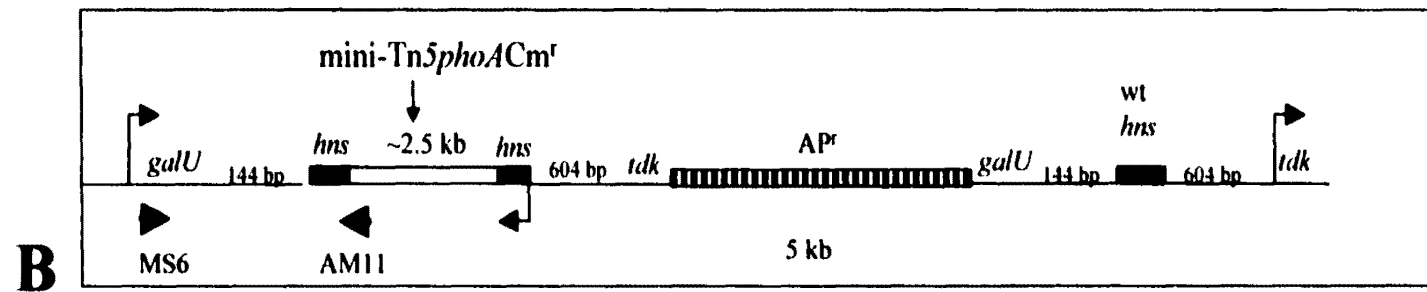
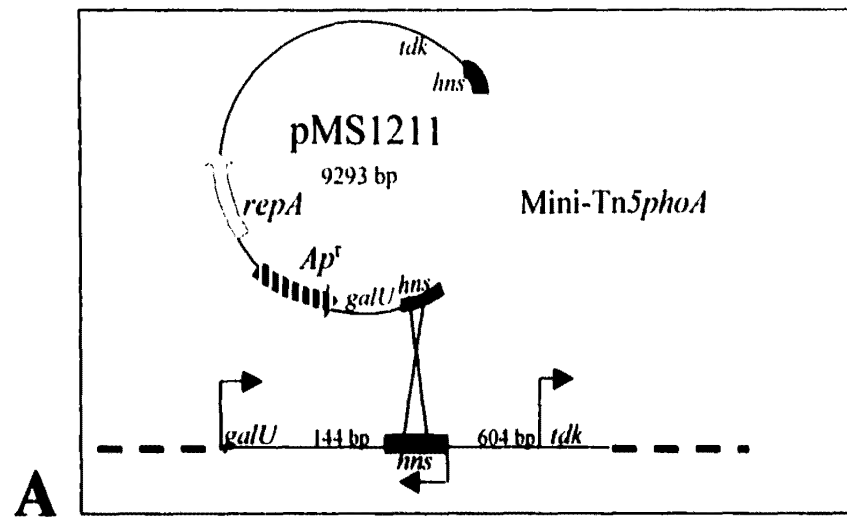


Figure 44. Allelic exchange following single-crossover between *hns* and flanking *galU* and *tdk* sequences, cointegration of pMS1211 into the S11 chromosome, and resolution to yield mutant S11366.15. Panel A represents the single-crossover event. Panel B shows cointegration of the plasmid pMS1211 that resulted in two chromosomal copies of *hns* with the distal 5' copy disrupted by the mini-Tn5*phoA*Cm^r insertion. Primer set MS6 and AM11 (large black arrow heads) were used to confirm that cointegration of the plasmid had occurred. In panel C MS6 and MS2 primers (large grey arrow heads) were used to confirm allelic exchange of the wild-type *hns* for *hns* that contained the transposon, in the resultant *hns* mutant S11G3C5. Black regions indicate *hns*. The hatched areas represent the ampicillin resistance gene. The clear areas show the mini-Tn5*phoA*Cm^r. Figures are not drawn to scale.

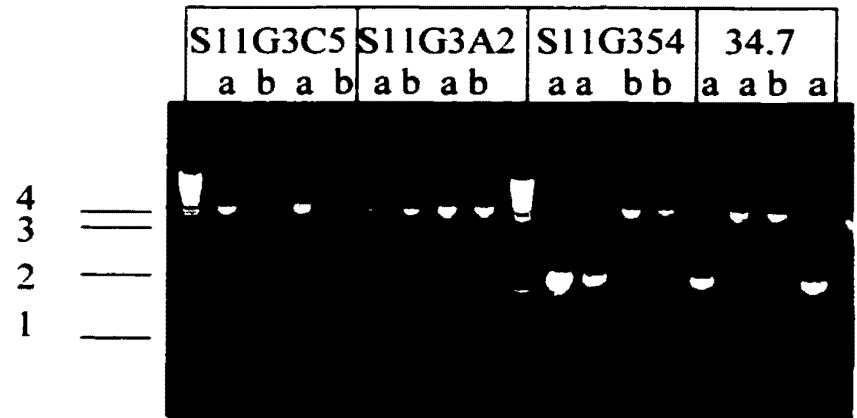


recombinants that were chloramphenicol resistant but ampicillin sensitive were isolated. Of the eight putative double recombinants isolated, only two strains (S11G3C5, S11G3A2) were confirmed to contain the predicted 4.5 kb fragment after PCR amplification (Figure 45, panel A) with the external primers MS6 and MS2 [Please note: two colonies of strains S11G3C5, S11G3A2 and S11G354 were tested simultaneously with both primer sets]. Strain S11G354 encodes wild-type *hns* on its chromosome and carries the suicide plasmid pMS1211. A 1.9 kb PCR product was predicted for S11G354 (wt copy) following PCR with primers MS4/MS5, but is not present, but the ~4.3 kb fragment amplified from the moderate copy number plasmid pMS1211 can be seen. One isolated colony of S11G3C5, S11G3A2, G3, E2, S11 and 34.7 were grown overnight, the DNA was isolated, restricted with *HindIII*, and then probed with *hns* by Southern blot analysis (Figure 45 panel B). Two fragments of DNA hybridized with the *hns* probe in the lanes that contained DNA from the two double recombinants (Figure 45 marked as S11G3C5 and S11G3A2 on panel B). However, only the 6.5 kb fragment had been predicted and was consistent with the 6.5 kb fragment obtained when restricted DNA from the original adherence mutant 34.7 was probed simultaneously. The DNA isolated from the LB broth cultures of the recombinants contained a mixed population of wild-type (9 kb band) and mutant (6.5 kb) because the *hns* probe hybridized to a 9 kb DNA fragment, and this 9 kb fragment corresponded to the size fragment noted when wild-type restricted DNA was probed with *hns* (Figure 45 panel B). Cointegrate restricted DNA (designated as G3 and E2) was included as a control and contained the predicted 6.5 kb and 4.3 kb size fragments. The 2 kb fragment in the lanes marked as G3 and E2 (cointegrates) corresponds to the *HindIII* restriction fragment generated from plasmid pMS1211 that contained *hns*::mini-Tn5*phoACm*^r. The Southern data

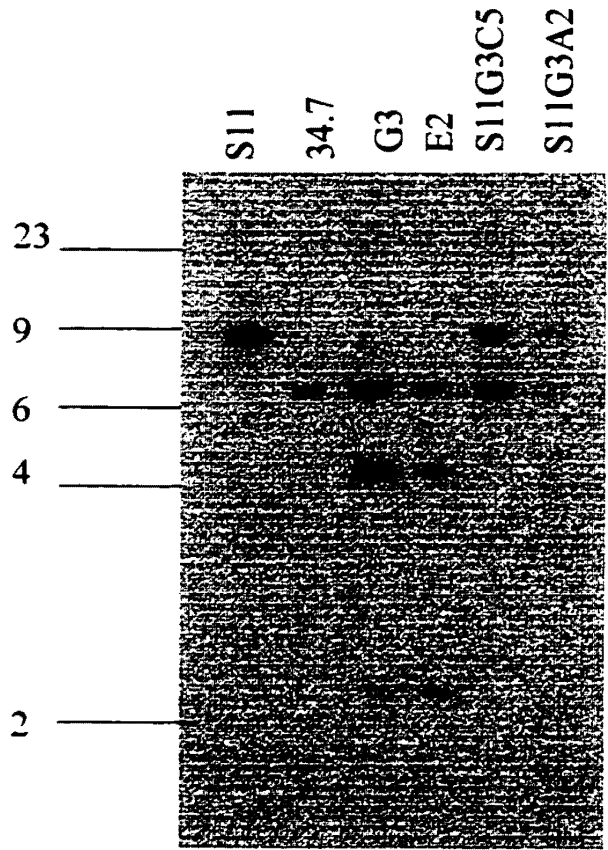
Figure 45. PCR and Southern hybridization analysis of two putative allelic exchange *hns* mutants S11G3C5 and S11G3A2.

Panel A: Ethidium bromide stained agarose gel electrophoresis of PCR fragments generated from strains S11G3C5, S11G3A2, S11G354, S11(wt) and 34.7 with the external primer set MS6/MS2 (designated as a) and the internal primer set MS4/MS5 (designated as b) [see Figure 41]. Primers MS6 and MS2 generate a 1.9 kb product from the parental strain S11(wt), ~4.5 kb product from mutants 34.7, S11G3C5, and S11G3A2. Primers MS4 and MS5 generate ~4.3 kb product from strains that carry pMS1211. Strain S11G354 served as the primer control, was grown only at 30°C, encodes wild-type *hns* on its chromosome, and carries plasmid pMS1211 (Please note: wild-type *hns* represented by a faint 1.9 kb band was present on the original electrophoresis gel, lanes marked as b under S11G354, but did not reproduce in the picture).

Panel B: Southern hybridization of *Hind*III-restricted total DNA from *hns*::mini-Tn5*phoAC**cm*^r mutants 34.7, S11G3C5, S11G3A2, parental S11 and cointegrates G3, E2. The wild-type *hns* gene cloned from B2F1 was labeled with ECL-horseradish-peroxidase and used as the probe in the analysis. Position of molecular weight size (kb) markers are indicated in both panels A and B.



A

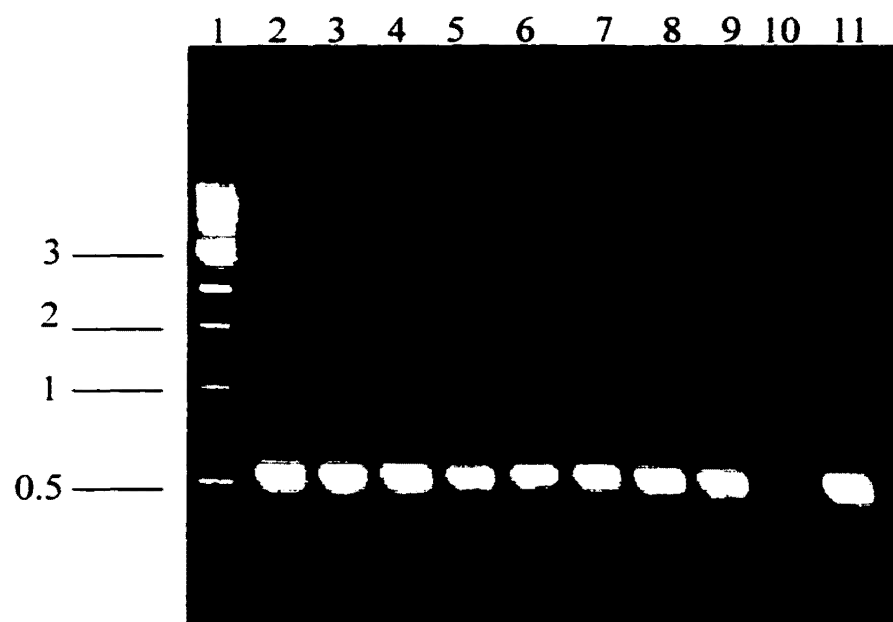


B

along with the PCR results suggested that double recombination had occurred and that the wild-type copy had been replaced by *hns::mini-Tn5phoACm^r* in some of the organisms within the population. However, the broth cultures contained some wild-type S11 DNA that was detectable by Southern analysis. The wild-type 9 kb band noted on Southern blots after overnight growth of S11G3C5 and S11G3A2, may represent isolates that deleted the mini-Tn5phoACm^r cassette from *hns* to yield a wild-type revertant. Instability of the insertion within *hns* encoded by the double recombinants S11G3C5 and S11G3A2 could be a reason for the discrepancy between the PCR and Southern data.

Mutants, S11G3C5 and S11G3A2, all of which grew well at 44 °C on chloramphenicol, were passaged more than 35 times in media without ampicillin. After these passages a sparse amount of growth of S11G3C5 and S11G3A2 was still evident in the first quadrant of agar that contained ampicillin. We speculate that during resolution of the plasmid from the cointegrate the wild-type copy of *hns* replaced the *hns::mini-Tn5phoACm^r* on the pSTAMP plasmid and was not cured. Indeed, isolates of the mutants that no longer grew on agar supplemented with ampicillin amplified (using primers AMP1 and AMP2 specific for the ampicillin resistance gene) a PCR DNA product that corresponded to the predicted 550 bp product derived from the ampicillin resistance gene (Figure 46). Wild-type S11 did not encode the ampicillin resistance gene and was always negative by PCR. Ultimately, approximately 200 isolated colonies of the mutants were tested by PCR and none could be found that did not contain the ampicillin resistance gene encoded by the plasmid. However, when plasmid preparations were made of the mutants, no plasmid was detected upon inspection by agarose gel electrophoresis nor did we

Figure 46. Ethidium bromide-stained agarose gel electrophoresis of PCR fragments generated from colonies of *hns::mini-Tn5phoACm^r* strains S11G3C5 and S11G3A2 with ampicillin specific primers AMP1 and AMP2. Lane 1 contained molecular weight markers, lane 2-5 and lane 6-9 included colonies of S11G3C5 and S11G3A2, respectively. Lane 10 contained strain S11 and lane 11 contained plasmid pSTAMP that encoded the ampicillin resistance gene.



detect the wild-type *hns* 1.9 kb fragment by PCR with MS6/MS2 primers. We decided that the results from growth of S11G3C5 and S11G3A2 on ampicillin plates and the plasmid isolation results were less conclusive than the PCR findings with primers AMP1/AMP2. Therefore, we surmised but could not conclusively prove that we had not cured S11/*hns*::mini-Tn5*phoACm*' (designated as S11G3C5 and S11G3A2) of the pSTAMP plasmid with wild type *hns*.

Partial inactivation of *hns* following insertion of a suicide plasmid. The construction of the temperature-sensitive suicide plasmid, pMS358, used to generate the *hns* mutation in S11, is detailed briefly below. PCR was used to amplify from the wild-type strain, S11 a 358 bp DNA product of *hns* that did not contain the promoter and was also missing 35 nucleotides from the 5' end and 19 nucleotides from the 3' end. PCR primers MS3 and MS9 were used and are shown in Figure 47. This truncated *hns* PCR product (358 bp) was ligated into the vector pCR2.1 to generate pCR358 (Figure 48). This DNA fragment was subsequently ligated into the *EcoRI* site of the temperature-sensitive suicide plasmid pSTAMP to generate plasmid pMS358(Figure 49). Plasmid pMS358 replicated well at 30°C but poorly at 44°C. The plasmid pMS358 was electroporated into S11 and transformants that were ampicillin resistant were recovered at 30°C. One S11(pMS358) colony was grown at 30°C, then stationary cultures were diluted (10-fold), plated and incubated overnight at 44°C on agar containing ampicillin. Nineteen putative cointegrates that were resistant to ampicillin at 44°C were isolated and screened by PCR to confirm that integration of the plasmid had occurred via a single crossover between the wild-type *hns* on the chromosome, and the *hns* fragment present on plasmid pMS358. Primers (MS10 and MS11) were used that hybridized to sequences on the chromosome external to the 5' and 3' ends of the *hns* coding region to determine if homologous recombina-

Figure 47. Primer set used to generate the 358 bp internal *hns* PCR product. The large black arrows marked MS3 and MS9 are the location of the primers that were used to amplify from wild-type S11 the *hns* 358 bp DNA fragment. The large white arrows are external primers (MS10 and MS11) that were used to confirm integration of the suicide vector that contained the 358 bp *hns* fragment into the S11 chromosome. Drawn to scale with the Clonemap program.

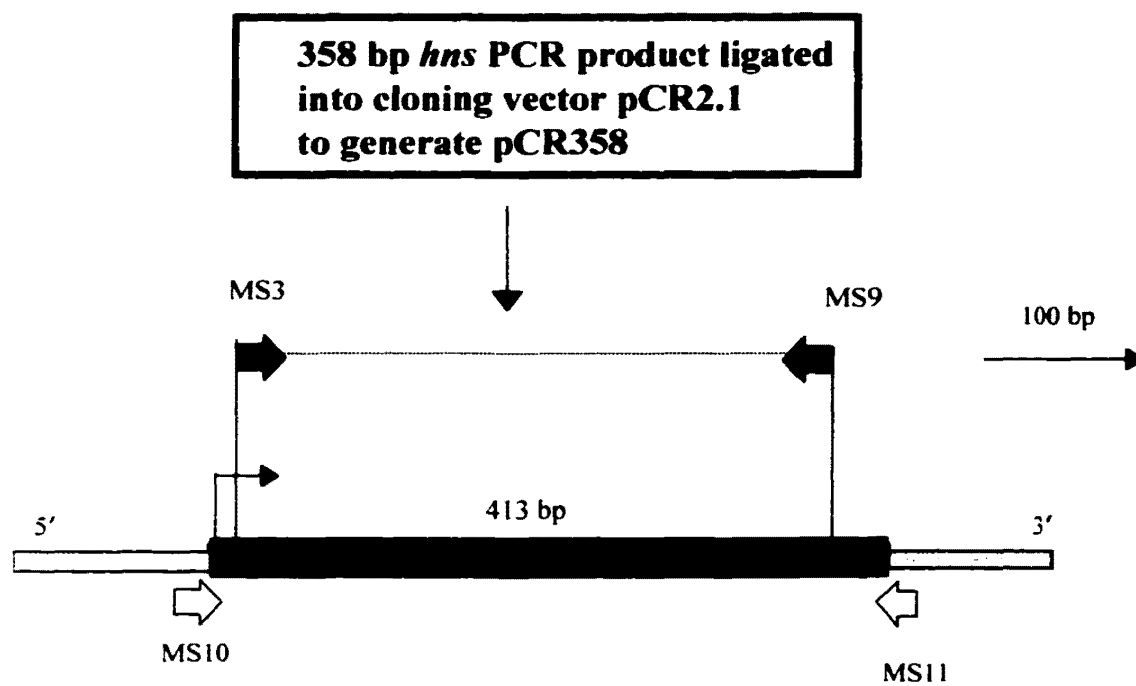


Figure 48. Plasmid map of pCR358. This plasmid contains the 358 bp product (small black region marked on the plasmid) used to construct the suicide plasmid pMS358. The vector pCR358 encodes kanamycin and ampicillin resistance.

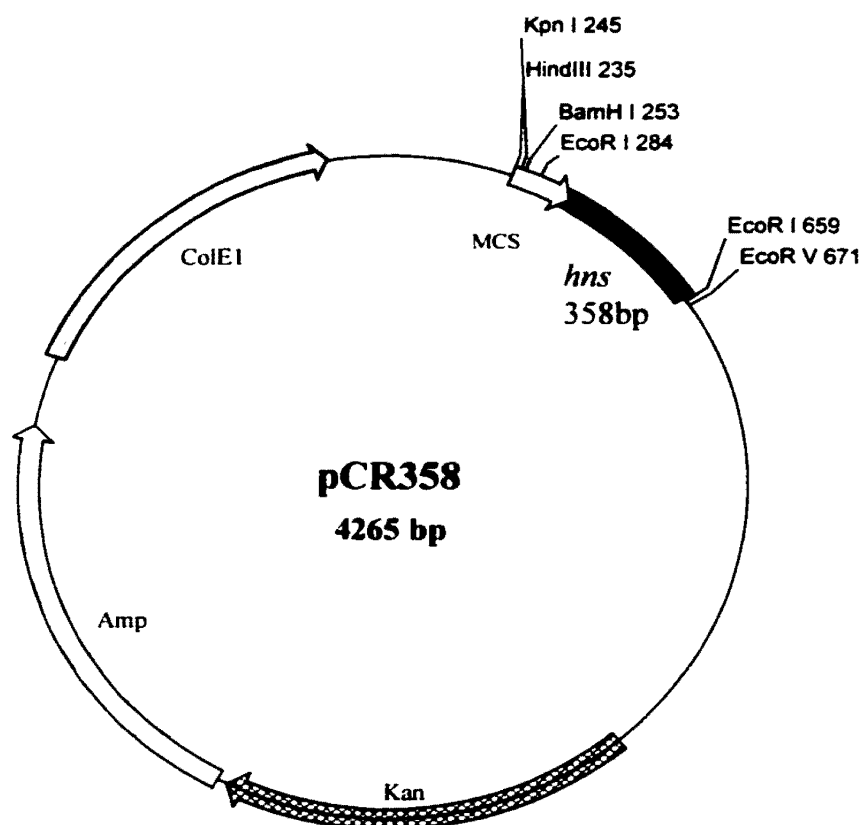
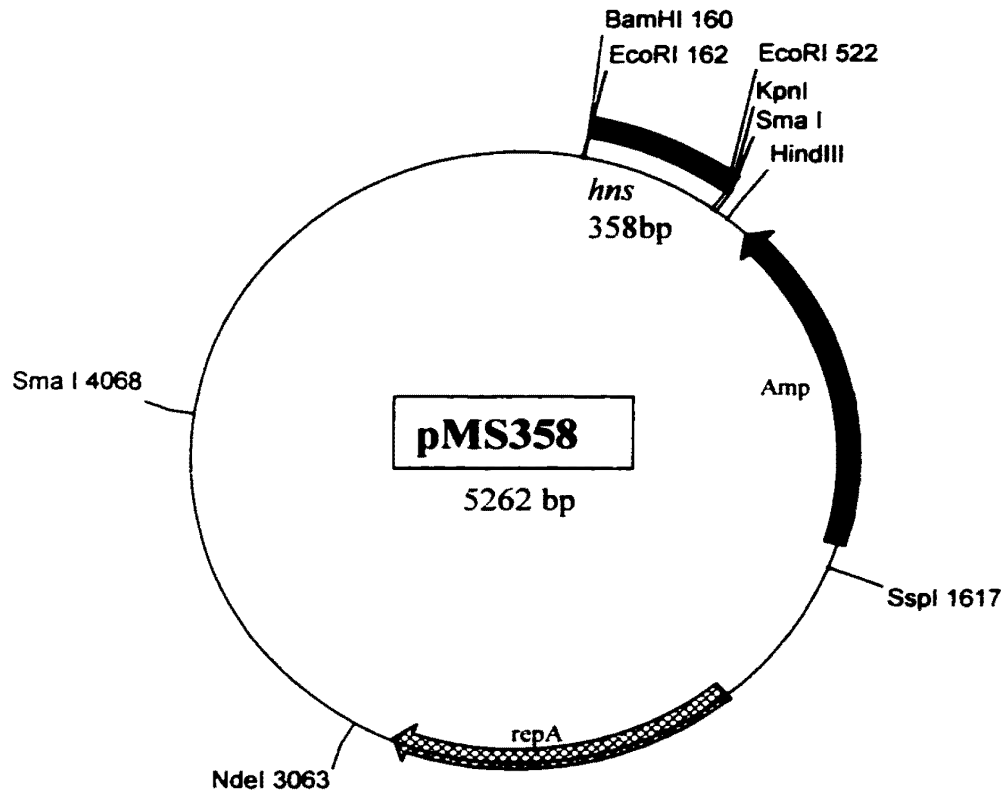


Figure 49. Plasmid pMS358. The small grey area represents the 358bp *EcoRI* fragment derived from pCR358 and ligated into the *EcoRI* site of pSTAMP to generate pMS358. The suicide plasmid contained a temperature sensitive origin of replication (*repA*), conferred ampicillin resistance, and was used to construct an insertional mutation within the *hns* gene of S11. Map is drawn to scale.



nation had occurred within *hns* (Figure 47). Four of the nineteen ampicillin-resistant colonies isolated at 44°C were confirmed to contain the plasmid insertion as evidenced by the presence of a 5.3 kb PCR product as compared to the 464 bp PCR product amplified from the wild-type S11 (Figure 50. Bands present in lanes 8-10 were faint and did not show up well in the picture). Southern blot analysis (Figure 51) of *Sall*-restricted total DNA isolated from cointegrates S11366.15 and S11366.19 hybridized to two fragments of size 6.8 kb and 4.0 kb, and *Bgl*II-restricted DNA hybridized to a fragment of 13 kb when probed with *hns*. Total DNA of the wild-type S11 was restricted with *Sall* and *Bgl*II, probed with *hns*, and hybridized to fragments that were 5.6 kb and 7.9 kb, respectively. The sizes of the fragments of restricted DNA from S11366.15 and S11366.19 that hybridized with *hns* probe correlated with the expected shift in size following the insertion of the 5.2 kb suicide plasmid within the *hns* gene.

Theoretically, insertion of the plasmid into the wild-type *hns* should generate two incomplete copies of the gene as illustrated in Figure 52. The copy of *hns* located 5' of the inserted plasmid encoded the native promoter but was missing 19 nucleotides from its 3' end (6 amino acids). The defective *hns* located 3' of the plasmid was missing 35 nucleotides from its 5' end (12 amino acids) and could not produce protein because it did not encode a promoter. However, because the *hns* that contained the native promoter was only missing 6 amino acids, we were concerned that some H-NS activity remained. Therefore, in addition to the adherence phenotype of the two mutants, the motility and alkaline phosphatase phenotypes (known to be affected by mutations in *hns*) were assessed.

Figure 50. PCR analysis of putative mutants of S11 that contain an insertion of the suicide plasmid pMS358 within *hns*. Ethidium bromide-stained agarose electrophoresis gel contained in Lanes 1, 2, 11 and 13 molecular weight markers; lane 3, strain S11(wt); lane 4, 34.7; lanes 8-10 and 14-15 strains that were ampicillin resistant at 44°C. External primer pair MS10/MS11 (Figure 47) was used to generate PCR products. Position of molecular weight (kb) markers are indicated.

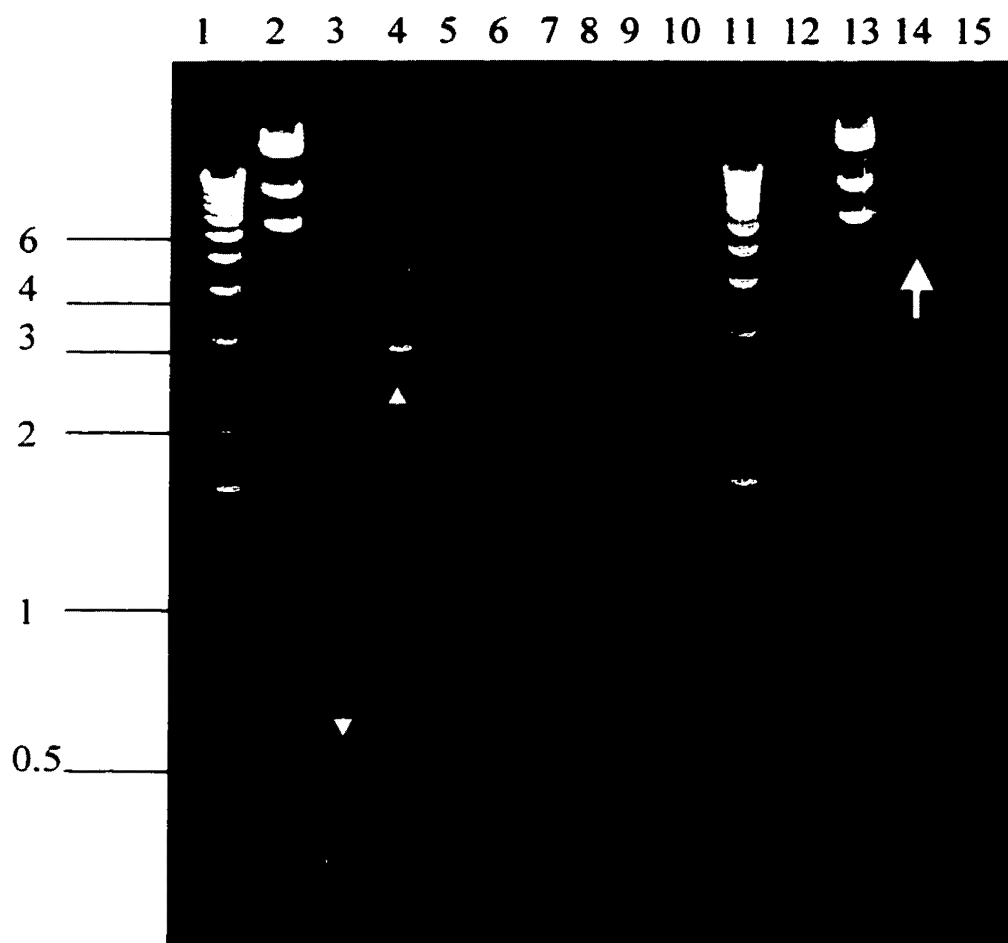


Figure 51. Southern hybridization of *Sal*I-digested and *Bgl*II-digested total DNA isolated from *hns* mutants S11366.15 and S11366.19. The *hns*::mini-Tn5*phoA*Cm^r mutant 34.7 and wild-type strain S11 were included as controls. Lanes that contain sample are: Lanes 2 and 9, parental S11; lanes 3 and 10, mutant 34.7; lanes 4 and 11, mutant S11366.15; lanes 5 and 12, mutant S11366.19. Plasmid pMS358, lanes 6 and 13. The wild-type *hns* gene cloned from B2F1 was labeled with ECL-horseradish-peroxidase and used as the probe in the analysis. The positions of the molecular weight (kb) markers, lanes 1 and 8, are indicated. Lane 7 is empty.

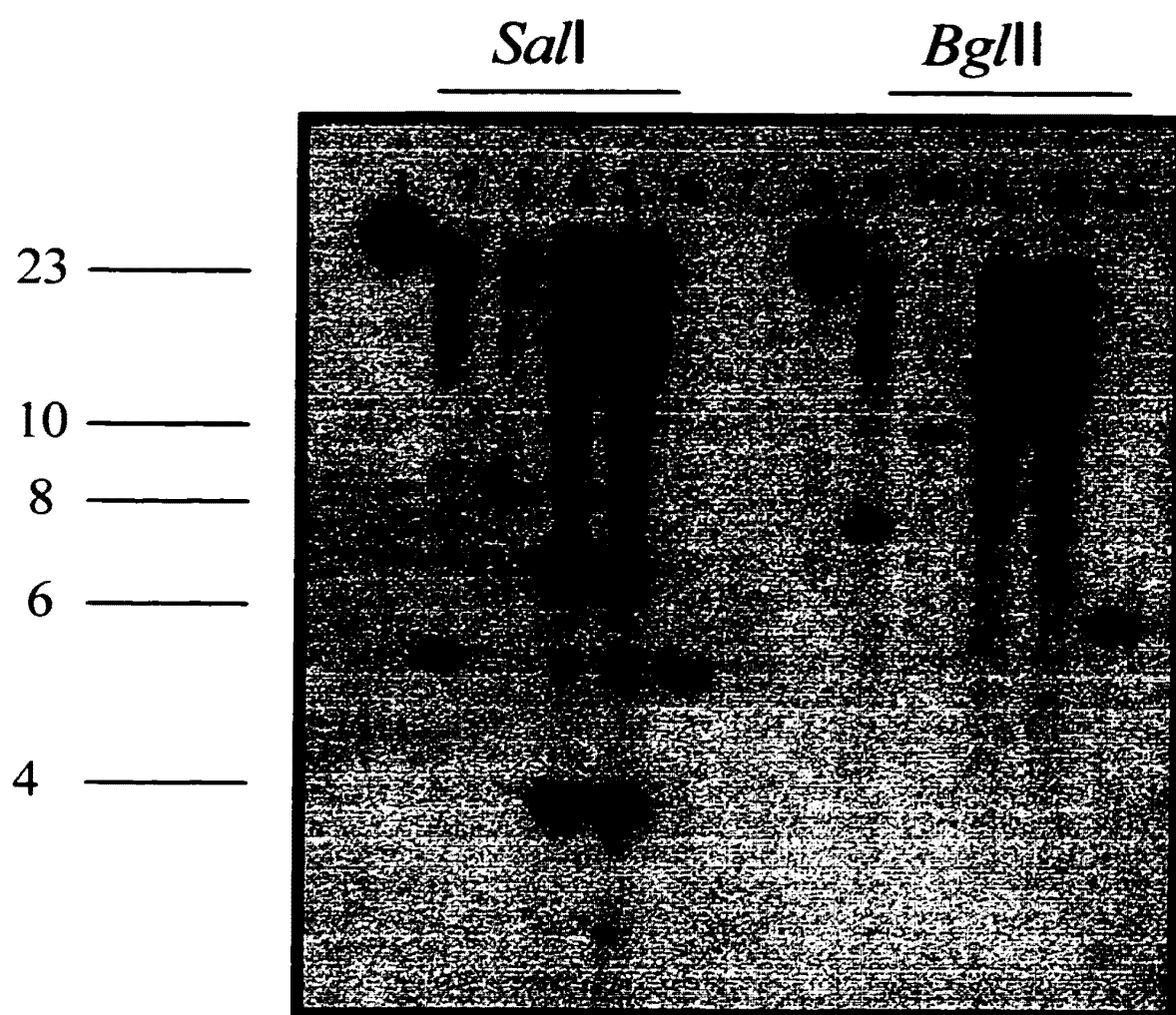
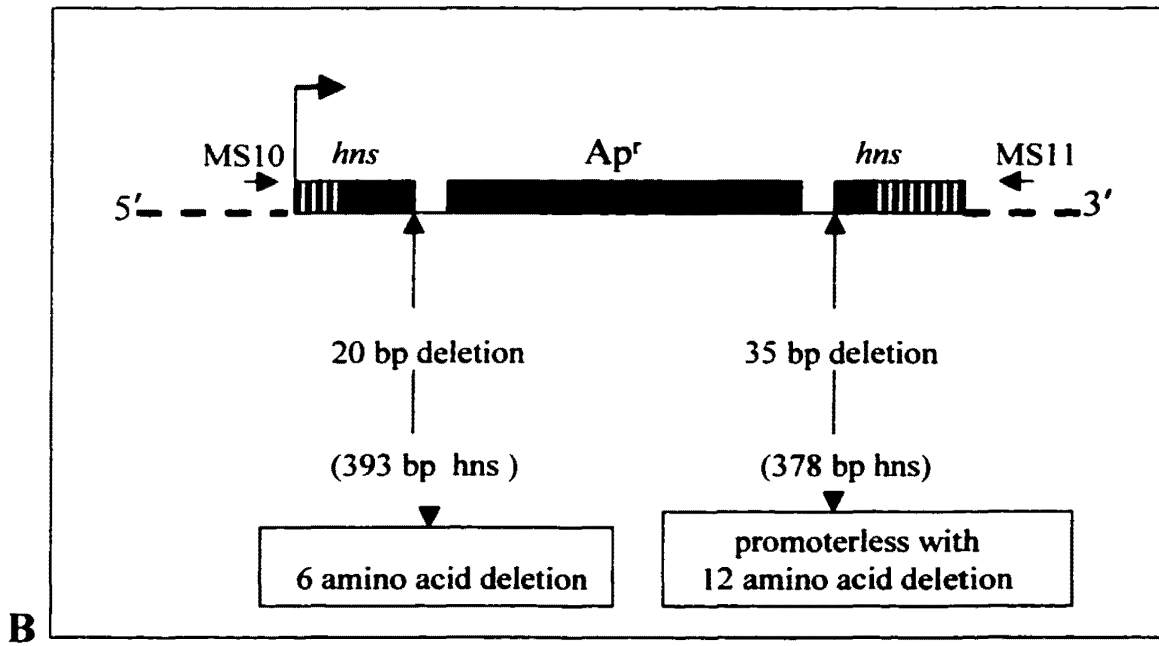
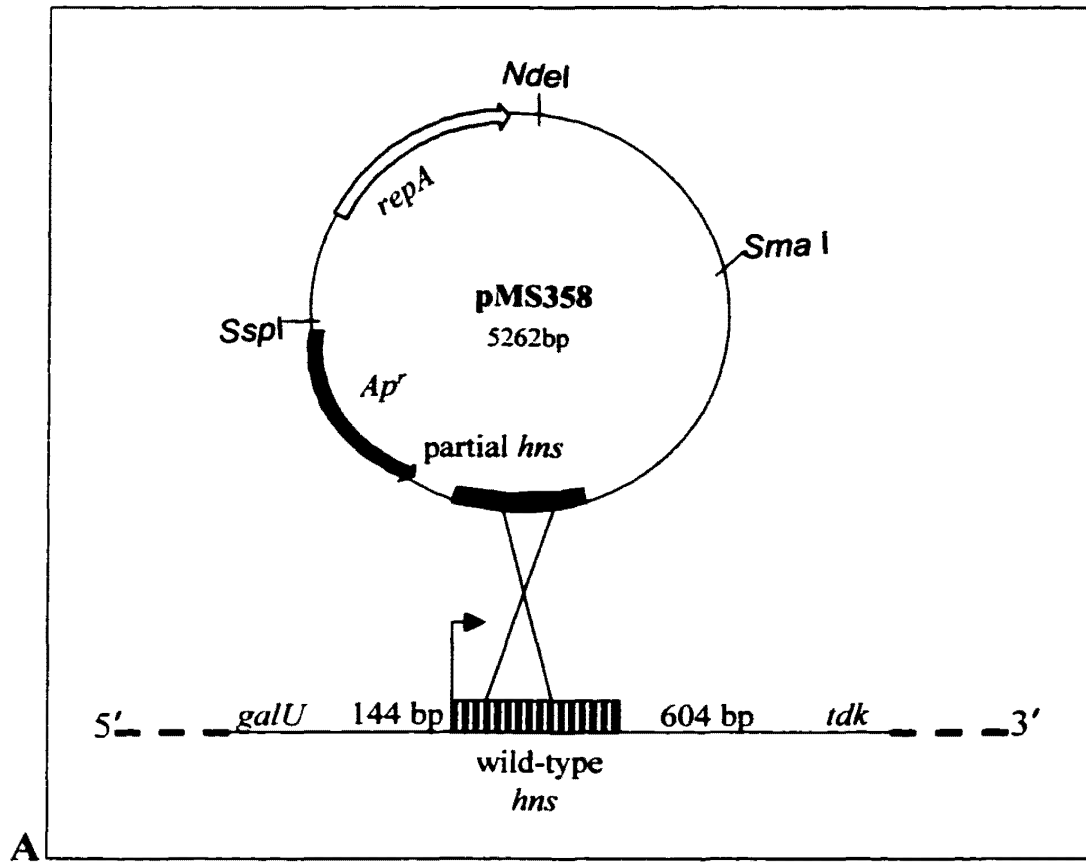


Figure 52. Illustration of the *hns* gene encoded by S11366.15 following insertion of the plasmid pMS358. Mutant S11366.15 encodes two incomplete copies of *hns* that has been disrupted by the 5.2 kb plasmid that contains the ampicillin gene (black regions). Primer set MS10 and MS11 hybridize to DNA sequences that are located outside of the *hns* gene (black small arrows) and were used to confirm that conintegration of the plasmid had occurred. The size of the PCR product predicted after insertion is ~5.6 kb and the predicted size for wild-type *hns* is ~500 bp. The grey regions represents the truncated *hns*. The hatched areas indicate wild-type *hns*. Panel A illustrates the single-crossover event between wild-type *hns* and the partial *hns*. Single-cross over between homologous *hns* sequences resulted in cointegration of the plasmid, shown in panel B.



XV. The effects of partial inactivation of H-NS in strain S11.

The *in vitro* growth rate of S11366.15 was similar to wild-type S11 when grown for eight hours at 37°C in LB broth (Figure 53). However, motility in semi-soft agar at 30°C was somewhat less than that of the parental S11 strain when the swarm diameters were compared (Figure 54 panel A). At 30°C in semi-soft agar, the diameter of the swarm ring of S11 was 16 mm as compared to 6 mm for mutant S11366.15 (Table 7). The original adherence mutant 34.7 that contained a transposon insertion within the *hns* gene, devoid of H-NS activity, was completely non-motile at 30°C in semi-solid agar. These results indicated that the activity of H-NS in mutant S11366.15 was not completely ablated but had been reduced. That alkaline phosphatase activity had been de-repressed when H-NS activity was reduced in mutant S11366.15 as indicated by the appearance of colonies on XP agar. Mutant S11366.15 was blue on media with XP after 18 hours of growth at 37°C as compared to wild-type S11 which was only faintly blue. However, the blue color of S11366.15 on XP agar was not as dark as the blue color noted for mutant 34.7 [mutant 34.7 which has no functional H-NS activity] (Figure 55).

In a standard three hour adherence assay, the capacity of S11366.15 and S11366.19 to bind to non-polarized T84 cells was reduced (Photomicrographs in Figure 56). Panels A and B show S11 with and without the suicide vector (pMS358), respectively. Mutant S11366.15 is shown in Figure 56 panel C and mutant 34.7 is shown in panel D. There was approximately a 10-fold difference in the number of adherent S11366.15 and S11366.19 organisms recovered ($\sim 10^5$ CFU/ml) as compared to $\sim 10^6$ CFU/ml bacteria recovered for wild-type S11 (Figure 57) following a 3.5 hour adherence assay on non-polarized T84 cells. In conclusion, the insertion of the suicide plasmid into S11 to generate S11366.15 and S11366.19 resulted in the reduced

Figure 53. Growth rates of *hns* mutant S11366.15 and 34.7 compared with parental strains S11 and B2F1. Cultures were grown for 8 hours with shaking in LB broth at 37°C.

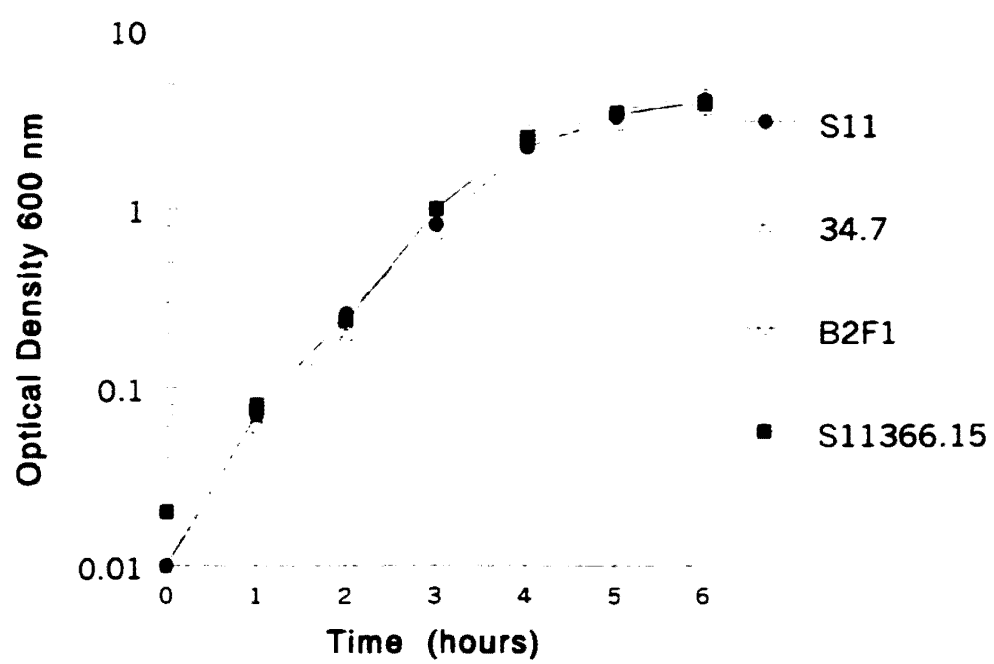
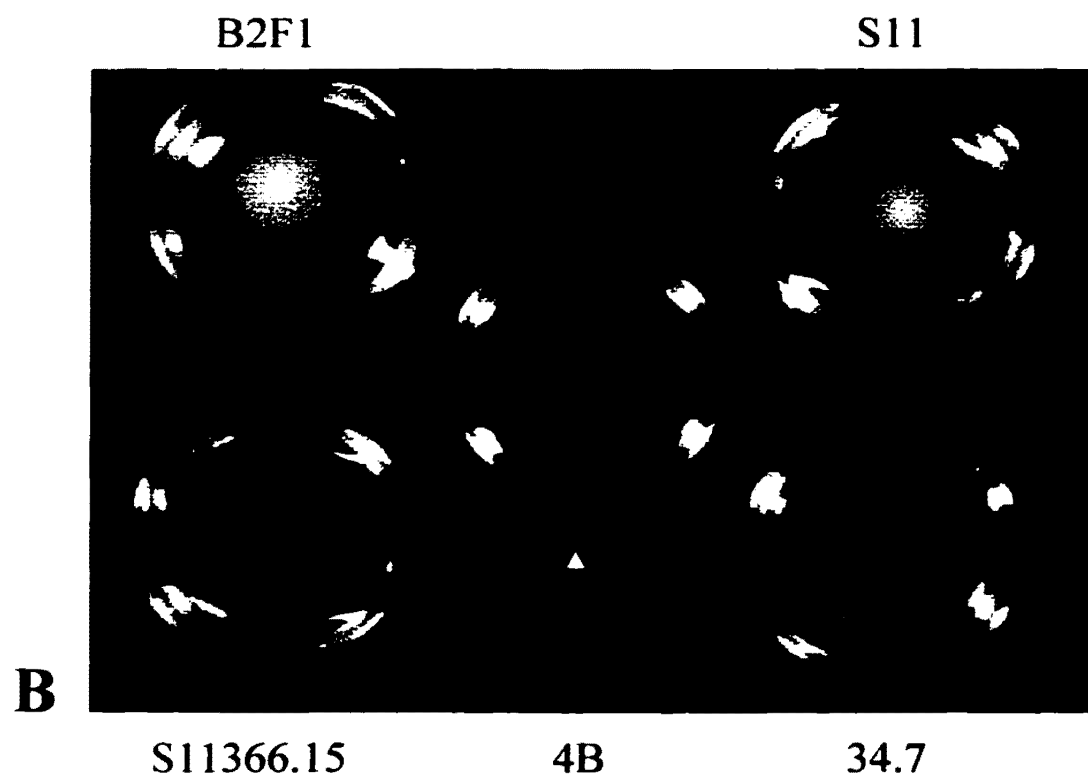


Figure 54. The motility of *hns* mutants S11366.15 and 34.7 compared to wild-type strains B2F1 and S11. Cultures were grown in semi-soft agar at 30°C for 15 hours. Panel B includes S11366.15(pTHK116) designated as 4B.



Footnote for Table 7.

^a Parental strains are STEC 091:H21 B2F1 and its plasmid-cured derivative S11. The isogenic mutant of B2F1 is 34.7. Mutant S11366.15 is the isogenic mutant of S11 and S11366.15.4B contains plasmid pTHK116-7 that encodes a functional *hns* gene.

^b Data are the mean values of three independent experiments done in triplicate.

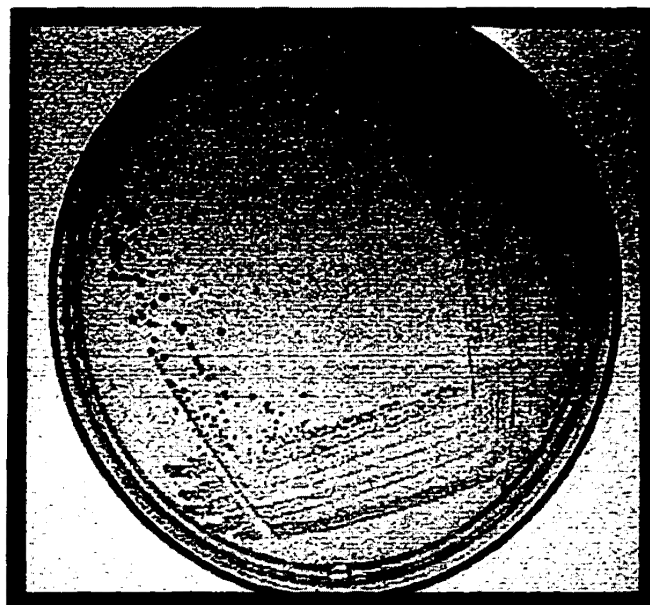
^c Values in parentheses represent the relative rate of motility of the isogenic mutant, S11366.15 and S11366.15.4B when compared to its parental strain. Motility rates were determined by comparing the swarm diameters of the *hns* mutant strains to those of the wild-type strains after 15 hours of growth at 30°C.

TABLE 7: Motility of parental strains B2F1 and S11 compared to isogenic mutants

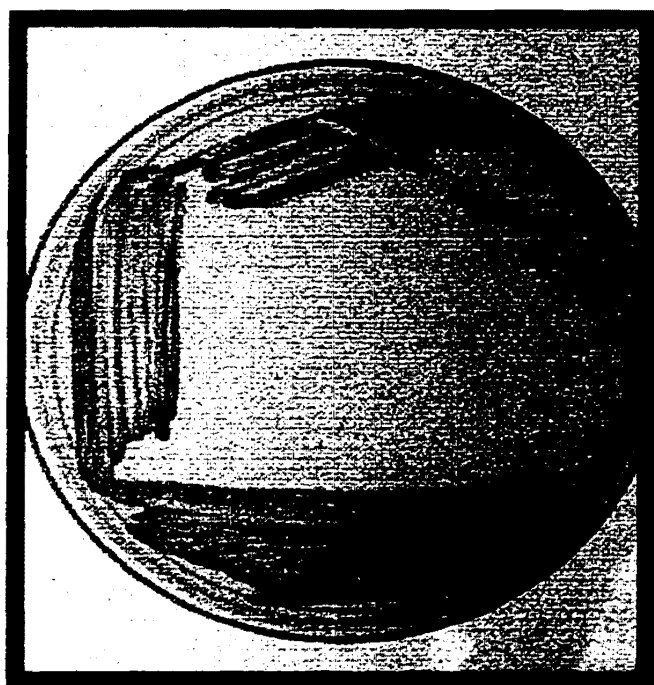
Strain ^a	Relevant genotype	Diameter of motility swarm ring (mm) ^b
B2F1	Wild-type <i>hns</i>	19.0
S11	Wild-type <i>hns</i> 90 kb plasmid cured	16.3
34.7	<i>hns::Tnp_hoA</i>	0
S11366.15	<i>hns::Ap^r</i>	6.0 (0.37) ^c
S11366.15.4B	<i>hns::Ap^r/pTHK116-7</i>	6.6 (0.41)

Figure 55. Appearance of *hns* mutants 34.7 and S11366.15 and wild-type strain B2F1 grown on XP agar. Strains were grown for 15 hours on agar that contained 80 µg/ml of the alkaline phosphatase substrate XP 37°C.

Wild-type B2F1



34.7 *hns::Tn5phoA*Cm^r
reduced adherence mutant



S11366.15 partial inactivation of *hns*
reduced adherence mutant

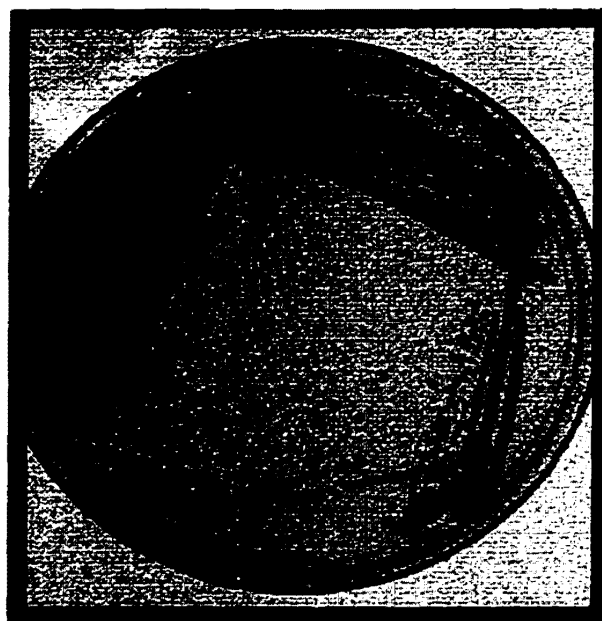


Figure 56. Adherence of *hns* mutants S11366.15 and 34.7 and the plasmid-cured wild-type strain S11 to T84 cells three hours post-infection. Panels A and B were infected with S11 and S11(pMS358), respectively. Mutant strains S11366.15 and 34.7 are shown in panels C and D, respectively. Black arrows mark clumps of adherent bacteria.

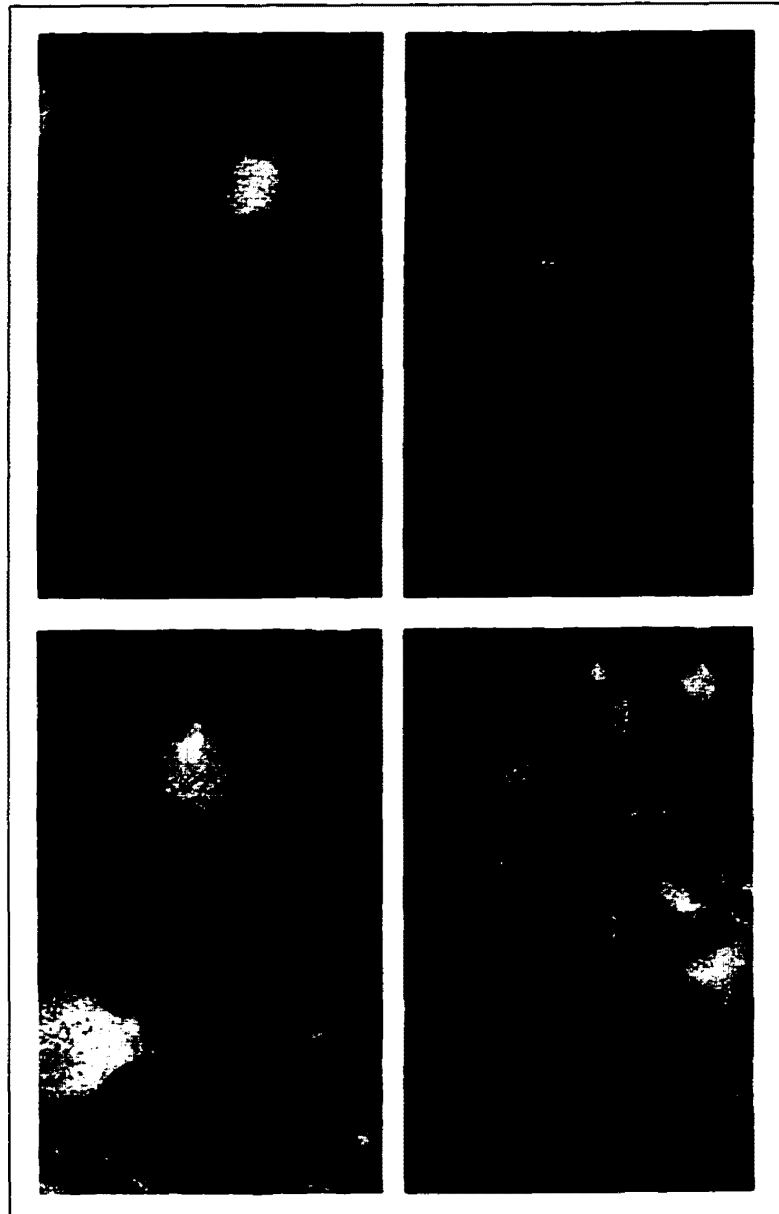
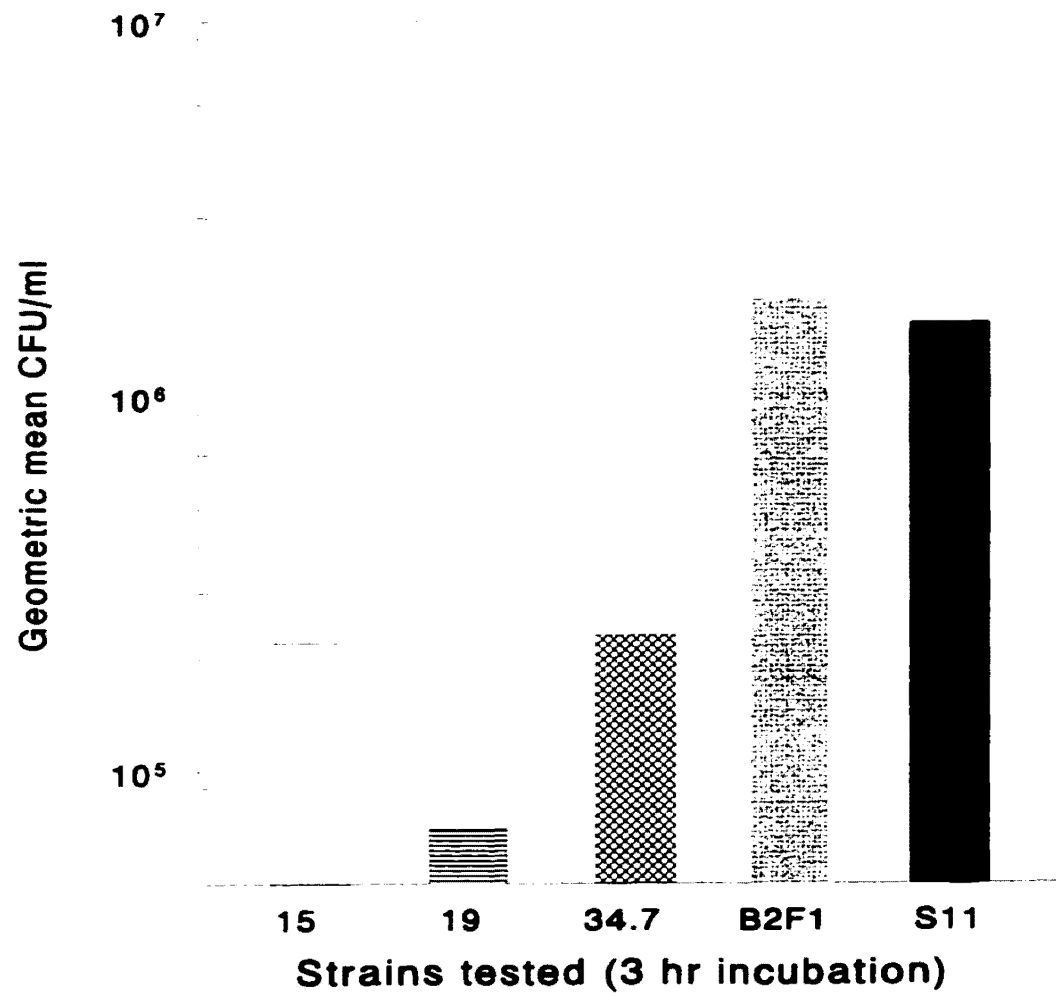


Figure 57. Non-polarized T84 cells infected with three *hns* mutants S11366.15, S11366.19 and 34.7 and wild-type strains S11 and B2F1. (Bars labeled as 15 and 19 represent mutants S11366.15 and S11366.19, respectively.) The relative number of bacteria adherent to non-polarized T84 cells after three hours of incubation was determined. Geometric mean values are plotted for each time point.

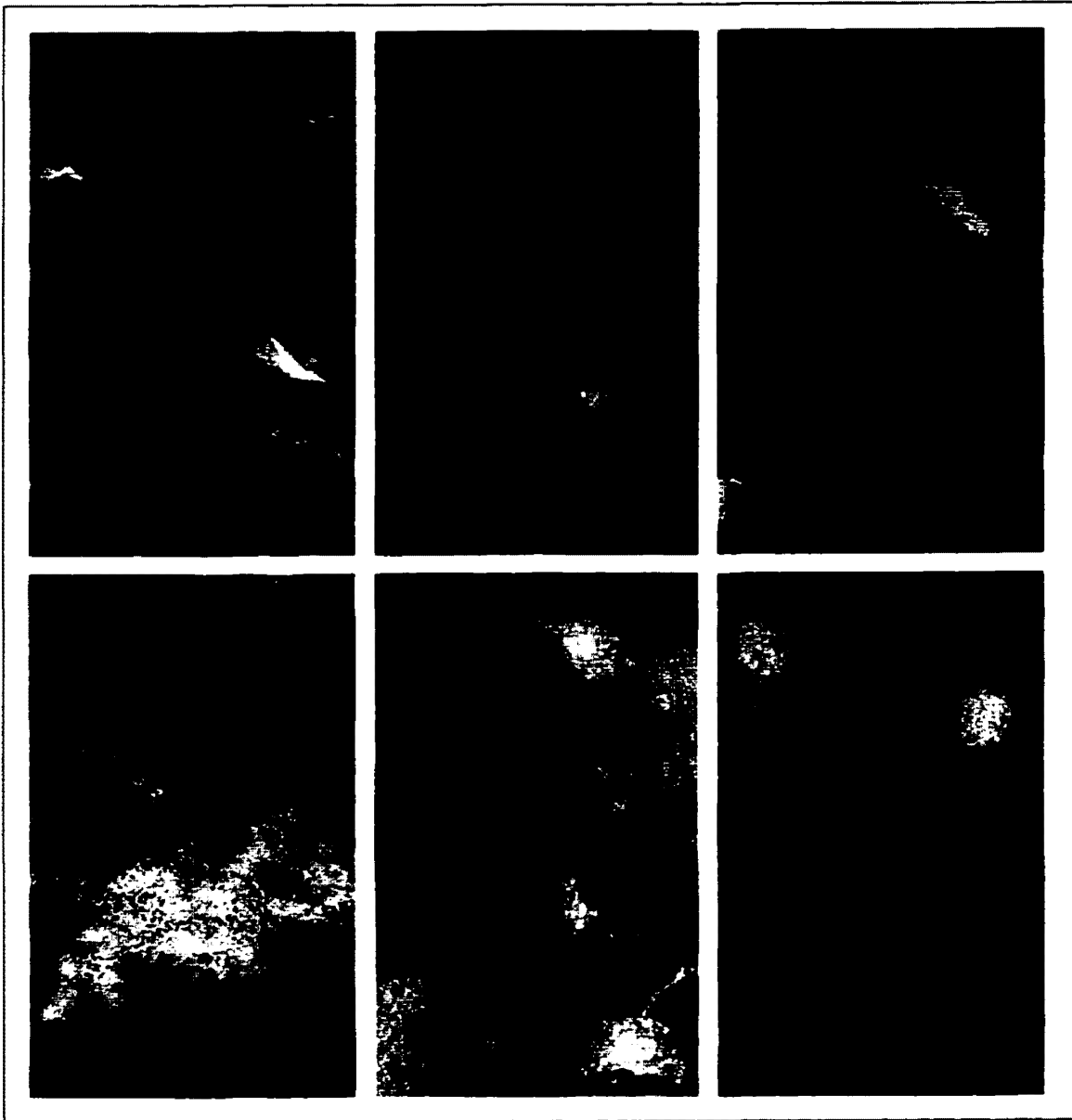


activity of the H-NS protein. The reduction in H-NS activity, indicated by a reduction in motility and an elevation of alkaline phosphatase, was sufficient to decrease the capacity of S11366.15 and S11366.19 to bind to T84 cells. Thus, the full function of H-NS is required for adherence of STEC 091:H21 strain S11 to T84 cells.

Presence of H-NS restores the wild-type adherence phenotype and the regulation of alkaline phosphatase to mutant S11366.15. The wild-type S11 strain adhered well to T84 cells but mutant S11366.15 did not (Figure 58 panels A and B, respectively). Microscopic observation revealed that when *hns*, carried on plasmid pTHK113 was introduced into mutant 34.7 the wild-type adherence phenotype was restored. Likewise, when S11366.15 was transformed with plasmid pTHK116-7, plasmid pACYC184 that encoded normal H-NS protein (gift from Dr. Tom Kawula, University of South Carolina), the adherence phenotype was restored to wild-type (Figure 58 panel F). Strain S11366.15 transformed with the pACYC184 vector alone was not adherent (panel D). S11 adherence was not adversely affected by the presence of plasmid of pACYC184 or pTHK116-7 (Figure 58 panels C and E, respectively).

Mutant S11366.15 that contained plasmid pTHK116-7 was no longer blue on XP media and, like wild-type, remained faint blue after 18 hours of incubation at 37°C (Figure 59). But, full motility was not restored to S11366.15 even in the presence of wild-type H-NS (Figure 54 panel B marked as 4B at white arrow). This partial reversion could be due to an *hns* gene dosage effect. A critical parameter might be the copy number of *hns*. Also, the partially inactivated form of the H-NS protein produced by mutant S11366.15 may form dimers with wild-type H-NS and lead to a decrease in H-NS activity resulting in a dominant-negative phenotype.

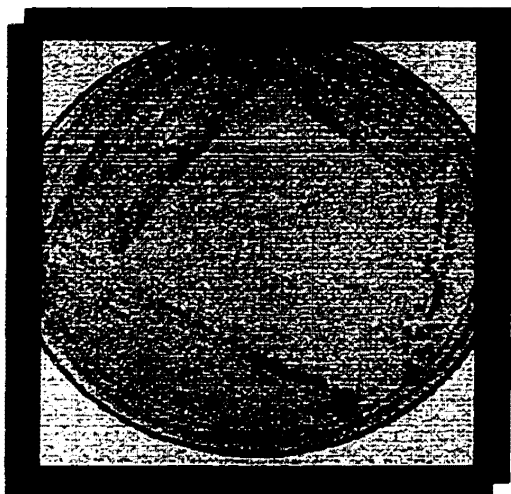
Figure 58. Restoration of the adherence phenotype of mutant S11366.15 to that of the parent strain S11 in the presence of wild-type H-NS. The photomicrograph shows: mutant S11366.15 alone. panel B; S11366.15(pACYC184), panel D and S11366.15 transformed with plasmid pTHK116-7, panel F. S11 is shown in panel A; S11(pACYC184), panel C and S11(pTHK116-7), panel E.



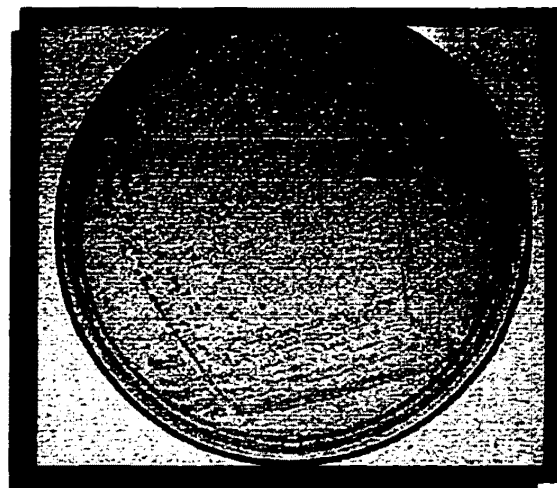
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Figure 59. The presence of wild-type H-NS restores regulation of alkaline phosphatase in the *hns* mutant S11366.15. Strain S11366.15.4B contains pTHK116-7. Strains were grown for 15 hours at 37°C on XP agar.

S11366.15.4B



Wild-type
B2F1



S11366.15

In conclusion, mutation of *hns* in a hemolysin-negative background showed that the absence of H-NS function, not the presence of increased STEC hemolysin, was responsible for the reduction in the adherence of mutant 34.7 to T84 cells. Therefore, the totality of evidence indicates that H-NS regulates the expression of an adherence factor (s) needed by B2F1 to bind to human colonic epithelial T84 cells *in vitro*.

Discussion

The initial aim of this project was to identify intestinal cell adhesins of the non-0157:H7, intimin negative 091:H21 STEC strain B2F1. Although that goal was not accomplished, three novel findings were generated from this dissertation project. First, we discovered that the H-NS protein positively regulates adherence of B2F1 to T84 human colonic epithelial cells. Second, we used a plasmid-cured strain of B2F1, S11, to show that the large plasmid is not required for the organisms to adhere to T84 cells at levels comparable to the parent B2F1. Third, we demonstrated that H-NS negatively regulates the plasmid-encoded hemolysin that is produced by B2F1. In the sections below, I discuss details of the areas of investigation that led to these three conclusions.

I. Adherence of STEC to human colonic epithelial cells in culture.

T84 cells were used to assess the capacity of STEC strain B2F1 to bind to human colonic epithelial cells. When B2F1 was applied to T84 cells, three hours were required before the number of adherent B2F1 was substantially higher (~2-3 logs) than the numbers of either control strain K-12 *E. coli* DH5 α or 86-4*eae* Δ 10 used. Therefore, the three hour period may be necessary, following environmental cues, to permit the expression by B2F1 of H-NS-mediated adherence factors. By comparison the EHEC strains tested (86-24 and H30) required a total of five hours incubation with T84 cells before the numbers of adherent bacteria present were greater than the poorly-adherent control strains. This observation is consistent with a previous report that

E. coli 0157:H7 required at least five to six hours of incubation before the adherence levels to HCT-8 (human ileocecal) or HEp-2 cells (human larynx) became statistically different from those of the *E. coli* K-12 strain (McKee and O'Brien, 1995). In that report the authors suggested that *de novo* protein synthesis or up-regulation of *E. coli* 0157:H7 bacterial proteins in the presence of the eukaryotic cell may account for this lag phase. Clearly the kinetics of adherence to human colonic epithelial T84 cells by intimin-positive and intimin-negative STEC are dissimilar and may be indicative of the different modalities by which these bacteria adhere to cells.

In this investigation, the intimin-positive *E. coli* strain 86-24 attached to polarized T84 cells in tight rounded clusters, and this phenotype was distinctly different from the irregular microcolonies noted on non-polarized cells. In contrast, the intimin-negative strain, B2F1, adhered in large irregularly shaped colonies to both polarized and non-polarized T84 cells. The marked difference between the adherence patterns of B2F1 and 86-24 to polarized versus non-polarized T84 cells could reflect the use by B2F1 and 86-24 of different intestinal cell adhesins. However, the intestinal epithelial cell receptor for intimin has not been identified. Evidence indicate that the EPEC intimin receptor may be an EPEC bacterial protein, named Tir (translocated intimin receptor), that is tyrosine-phosphorylated following insertion into the eukaryotic cell membrane (Rosenshine et al., 1996). Tir is also encoded by 0157:H7 but the Tir protein produced by 0157:H7 has not been shown to be phosphorylated following insertion into epithelial cells (Ismaili et al., 1998). Furthermore, purified intimin and purified intimin-maltose binding protein fusions can adhere to epithelial cells in the absence of Tir (Kaper et al., 1998). In addition, reports by Frankel et al. show that EPEC and EHEC intimin can bind to a subset of

β_1 integrins (Frankel et al., 1996). Therefore, the Tir protein may not be the only receptor employed for 0157:H7 (e.g. strain 86-24 used in this study) adherence to epithelial cells and 0157:H7 may well utilize eukaryotic encoded receptors. Thus, the change in the phenotype of 86-24 on polarized T84 cells may reflect a re-distribution of the intimin-specific receptors that occur after polarization. A similar re-distribution of B2F1-specific receptors does not appear to occur.

The qualitative adherence data usually supported the quantitative data for all strains tested. For example, the number of *E. coli* DH5 α recovered in a quantitative assay probably reflected the amount of bacteria attached to the plastic slide since bacteria were seen by light microscopy to be attached to plastic and only rarely to the monolayer. This basal level of adherence was detected for poorly adherent strains whenever quantitative analysis was done, and it was considered to reflect non-specific attachment of bacteria. In contrast, although wild-type B2F1 attached to the plastic slides they also adhered to T84 cells in large clusters unlike DH5 α . The number of adherent EHEC and STEC recovered after 5 hours of incubation were comparable and microscopic assessment of the adherence capacity of wild-type revealed clusters of bacteria adherent to T84 cells.

The plasmid p0157 encoded by *E. coli* 0157:H7 strain 933 has been the subject of numerous investigations on its role in adherence (Karch et al., 1987; Tzipori et al., 1987; Toth et al., 1990). Because none of the previous published reports dealt with STEC intimin-negative strains, we sought to determine if the large plasmid played a role in the adherence of B2F1 to T84 human colonic cells. In our first experiment, the data indicated that the large plasmid alone was not sufficient to impart the wild-type adherence phenotype to a non-adherent laboratory

strain of *E. coli* DH10B. In a second experiment, a plasmid-cured derivative of B2F1 designated as S11, when assessed by quantitative and microscopic analysis, adhered to T84 cells as well as wild-type B2F1. Together these two experiments indicate that the large plasmid encoded by STEC does not encode all the factors necessary for adherence of these bacteria to intestinal epithelial cells *in vitro*. However, these experiments did not establish whether or not the ~90 kb plasmid is important for *in vivo* colonization of STEC in a suitable animal model. The role of the large plasmid in pathogenicity or virulence has not been clearly defined in large part because of a lack of an appropriate animal model. The available models address particular aspects of STEC-associated disease. For example, the streptomycin-treated mouse model has been used to show that both *E. coli* 0157:H7 strain 933 and its plasmid-cured derivative 933-cu are maintained at a stable level when they are individually fed to mice (Wadolkowski et al., 1991). But when the two strains are fed simultaneously, 933-cu is unable to compete with the parental strain in two-thirds of the mice tested (Wadolkowski et al., 1991). In 1993, Lindgren et al. reported that STEC B2F1(Str^r) could colonize the large intestine of orally infected streptomycin-treated mice as well as EHEC 933 (Str^r). In addition, the plasmid-cured derivative of B2F1, S11, also colonized as well and was as virulent as the parent strain, B2F1 in this streptomycin-treated mouse model of infection (personal communication from Dr. Angela Melton-Celsa). Co-colonization studies in this model with strains B2F1 and S11 have not been done, so it is not known whether the absence of the large plasmid would reduce the capacity of S11 to maintain colonization in the presence of the parent. B2F1 is highly virulent in the streptomycin-treated mouse model and virulence is largely due to the production of Stx2d toxin (Lindgren et al., 1993). Therefore co-colonization of two strains (S11 and B2F1) capable of producing similar

amounts of activatable toxin may not be feasible because the host could be killed so quickly that colonization can not be adequately assessed.

The gnotobiotic pig model was used extensively to establish the essential role of the *eae* gene in A/E lesions of *E. coli* 0157:H7. In that model, the large plasmid carried by 0157:H7 strain 933 does not contribute to the colonizing capacity of the strain (Tzipori 1987). An initial report of infection of gnotobiotic pigs with B2F1 indicated that it did not colonize those animals or cause disease. By contrast, intimin-positive *E. coli* 0157:H7 strains were shown to colonize gnotobiotic pigs. Strain 86-24*eae*Δ10, that contained an internal deletion in *eae*, did not colonize and persist when fed to gnotobiotic pigs (McKee et al., 1995). However, when strain 86-24*eae*Δ10 (no functional intimin is produced) was fed to streptomycin-treated mice the *eae* deletion mutant still colonized these animals. Thus, the streptomycin-treated mouse model could not be used to assess colonization of STEC strains that utilize intimin as their primary adherence factor. In fact, *E. coli* K-12 strains adhere to and colonize the mouse intestinal tract but at lower levels than does 86-24. A preliminary experiment (Dr. Evelyn Dean-Nystrom, USDA, personal communication) with caesarean-derived-colostrum-deprived (CDGD) piglets revealed that B2F1 did colonize the large intestine of piglets. As predicted, the 0157:H7 strains, but not B2F1, caused A/E lesions. Since intimin-negative STEC strain, B2F1, can colonize CDGD piglets, this may be a more relevant model than the streptomycin-treated mouse model to assess colonization of the large intestine by STEC.

II. Comparison of important characteristics exhibited by adherence mutants of strain B2F1.

Only three adherence deficient mutants (34.3, 34.7 and 30.10a) were isolated as a result of transposon mutagenesis of B2F1 and none of the three adherence mutants that were isolated contained the transposon insertion within the structural gene of the adhesin encoded by B2F1. We reasoned that if B2F1 encoded more than one adhesin it would have been difficult, using the current method, to isolate a null adherence mutant of B2F1. Mutants that exhibited an obvious reduction in their capacity to adhere to T84 cells were chosen for further study. Therefore, the criteria used to choose mutants may have been too stringent and eliminated other candidates.

Southern hybridization analysis of the three mutants revealed that 30.10a contained the insertion in a gene that was carried on the ~90 kb plasmid. Because the large plasmid did not appear to encode genes essential for adherence of B2F1 to T84 cells (see above) we sought an explanation for the poor adherence phenotype of that mutant and the fact that in a 5 hour adherence assay the T84 monolayer was destroyed. Therefore, the STEC hemolysin phenotype of mutant 30.10a was tested. Abnormally large zones of lysis around colonies were produced when the isolate was grown on agar supplemented with SRBCs. However, when the protein profile of mutant 30.10a was analyzed by SDS-PAGE, the amount of STEC hemolysin protein produced was not elevated. Separate reports by Schmidt et al. (1994) and Bauer et al (1996) support the theory that in mutant 30.10a export of STEC hemolysin into the supernatant may have occurred because the mini-Tn*5phoAC*^{m^r} disrupted a gene or cis-regulatory element (not yet described) on the large plasmid that regulates the STEC hemolysin translocation genes *hlyBD*. Specifically, Schmidt et al., (1994) reported that plasmid pEO40 (that contained a 11.9 kb *Bam*HI fragment that encoded the entire STEC *hlyCABD* operon cloned from the ~90 kb plasmid p0157 onto pUC8) exhibited strong hemolytic activity when expressed in an *E. coli* laboratory

strain C600 (140-fold higher than when expressed by EDL933). When the entire ~90 kb plasmid was transformed into strain C600, no hemolysin activity was detected in the supernatant (Bauer and Welch, 1996), and the difference in expression was partially overcome by adding the translocator genes *hlyBD* in trans. In sum, we propose that the mini-Tn5*phoACm'* insertion into the plasmid of mutant 30.10a altered transport of STEC hemolysin which affected adherence of the organism to T84 cells.

STEC hemolysin is translocated by two membrane-associated proteins (HlyBD) that are normally synthesized at a lower level than cytosolic HlyC and the precursor form of HlyA (Koronakis and Hughes, 1996). HlyB is an inner membrane protein which has an integral membrane domain that consists of six helical transmembrane segments and HlyD contains a transmembrane segment that spans the inner membrane of the cell (Koronakis et al., 1995). In addition to HlyBD, an unlinked chromosomally encoded outer membrane protein, TolC, is required for translocation of STEC hemolysin and is believed to form a porin-like structure to create a periplasmic bridge to components of the translocation HlyBD complex (Koronakis et al. 1997). We reasoned that if the concentration of HlyBD and TolC proteins are substantially increased due to a defect in the regulation of these proteins, the increased expression of HlyBD and TolC could affect the normal concentration and distribution of other proteins (such as an adhesin) that are normally exported to the outer membrane of the cell. Indeed, the adhesin expressed by 30.10a may not be able to assemble correctly on the outer membrane of the cell when abnormally high levels of HlyBD and TolC are expressed. Consequently, 30.10a does not adhere to T84 cells with a wild-type phenotype but binds inefficiently as single bacterium to T84 cells.

Two other adherence mutants, 34.7 and 34.3, that did not adhere to T84 cells were characterized, and Southern restriction mapping and hybridization with the mini-Tn5*phoA*Cm^r specific chloramphenicol resistance gene probe revealed that these two mutants were clonally related. Therefore, we selected 34.7 for further study. Subsequently, the gene that contained the transposon insertion was cloned and identified as the histone-like nucleoid-structuring gene (*hns*). In 34.7 the disruption of *hns* by mini-Tn5*phoA*Cm^r occurred early in the nucleic acid sequence of the gene, and eliminated production of H-NS. The *hns* gene encodes a cytoplasmic protein that has been previously shown by other investigators to be important in the regulation of the expression of a variety of unrelated genes encoded by *E. coli*. (reviewed by Atlung and Ingmer, 1997). As mentioned earlier, mutant 34.7 was blue within 15 hours when grown on LB agar that contained XP and had been presumed to contain a positive PhoA fusion. However, sequence data revealed that the transposon insertion occurred out-of-frame within the *hns* gene; therefore, no functional PhoA could have been expressed from the *hns* promoter. One explanation for the observation that the 34.7 *hns* mutant was blue at 15 hours on XP media is that H-NS, known in most cases to act as a repressor, may downregulate endogenous levels of PhoA. Indeed, other investigators have inadvertently isolated mutants that contained Tn*phoA* insertions within the *hns* gene because these mutants were blue on media that contained XP and were believed to contain positive PhoA gene fusions (Harrison et al., 1994; Ciacci-Woolwine et al., 1998). In one such example Harrison et al., (1994) isolated an out-of-frame, blue-on-XP agar *hns*112::Tn*phoA* mutant of *Salmonella typhimurium* strain C5 that was attenuated for virulence in a mouse model.

The *hns* mutant 34.7, like the 30.10a mutant was altered in its hemolysin phenotype. In contrast, the total amount of hemolysin produced by 34.7 was increased relative to levels produced by the parent strain B2F1. Nevertheless, 34.7 did not cause discernible damage to T84 cell monolayers after five hours of incubation, in contrast to 30.10a. Even though 34.7 secreted STEC hemolysin, hemolytic activity was low with an average titer of 2 detected in culture supernatants 5 hours after infection of T84 cells. So, the amount of STEC hemolysin in solution may not have been sufficient to cause overt damage to T84 cells during the 5 hour adherence assay. Indeed, studies by Bauer and Welch (1996) with *E. coli* 0157:H7 hemolysin concluded that the amount of concentrated STEC hemolysin needed to effectively kill bovine BL-3 cells (30%) was ~70 ng. Hence, it is unlikely that 70 ng of STEC hemolysin were present in the supernatants recovered 5 hours post infection of T84 cells by mutant 34.7. The concentration of STEC hemolysin in the supernatants was not measured. However, when supernatants recovered from T84 cells infected for 5 hours with 34.7 were tested for hemolytic activity by tube assay with SRBCs as the indicator cells, no SRBC lysis was evident. Moreover, *hns* encoded by the hemolysin negative strain S11 (plasmid-cured derivative of B2F1) was partially inactivated by insertion of a suicide plasmid into the *hns* gene. Consequently, this *hns* mutant (S11366.15) produces an H-NS protein missing 6 amino acids at its carboxy-terminus, a region that contains domains important in both DNA-binding (Dorman et al., 1999; Bertin et al., 1999) and protein-protein interactions (Donato et al., 1999). Indeed, the *hns* mutation in S11366.15 rendered the non-hemolytic strain unable to bind to T84 cells. This finding directly indicates that H-NS serves as a positive regulator for STEC adherence to T84 cells in culture, and indirectly shows

that undetectable hemolysin-mediated damage to T84 cells by mutant 34.7 was not responsible for the poor adherence of that mutant.

In addition, the motility of mutant 34.7 was examined because previous observations by other groups indicate that H-NS is involved in the biogenesis of flagella in *E. coli* (Bertin et al., 1994; Hinton et al., 1992). Specifically, H-NS affects expression of flagella biosynthesis at the level of the flagellum-chemotaxis master regulon encoded by *flhCD* (Bertin, et al., 1994). Subsequently, we found that mutant 34.7 was nonmotile at 30°C, whereas the parent, B2F1, was motile at 30°C. Mutant S11366.15, that expresses a partially inactivated form of H-NS, exhibited a defect in its capacity to swarm when grown at 30°C. In addition, clinical strains of *E. coli* 0157:H⁻ (Gunzer, et al., 1992) as well as STEC OX3:H⁻ (Bonnet et al., 1998) that do not express flagella have been isolated from the stools of patients with HUS. Clonal analysis revealed that the non-motile strain of serotype OX3:H⁻ is related to OX3:H21, and that both isolates belong to STEC group 1 (Whittam, 1998; Bonnet et al., 1998). Hence, flagella-negative strains of EHEC and STEC that have “silenced” the expression of flagella are fully capable of colonizing and maintaining infection of the human intestine. Therefore, the nonmotile status of mutant 34.7 is probably not the cause for its reduced adherence to human colonic T84 cells.

III. Implications of the current investigation and further studies warranted.

Mutations in *hns* are pleiotropic, affecting the synthesis of an array of gene products involved in numerous biological processes in *E. coli* (Laurent-Winter et al., 1997; Atlung and Ingmer, 1997; Donato and Kawula, 1999;). For example, H-NS acts as a repressor of *fimB*

(encodes the type 1 fimbrial subunit protein) expression by directly binding to the promoter region and inhibiting transcription (Donato et al., 1997). In *hns* mutant strains that carry *fimB-lacZ* operon fusions, expression of β -galactosidase is 30-fold higher relative to that of the wild-type H-NS expressing strains (Donato and Kawula, 1999). Similarly, results of the present study indicate that H-NS represses the level of STEC hemolysin that is typically produced and secreted by wild-type B2F1 since mini-Tn5 *phoAC* Cm^r disruption of *hns* in 34.7 resulted in an increase in hemolysin production. In the same mutant, adherence to T84 cells was reduced at 37°C and no swarming was evident at 30°C. These three phenotypes appear to be linked to the *hns* null mutation that is present in mutant 34.7 and suggests that H-NS coregulates expression of gene(s) important in adherence as well as motility and hemolysin production in STEC.

We theorize that for STEC to adhere to colonic epithelial cells, expression of hemolysin is repressed or tightly controlled. In this study, 5 hours post-infection of T84 cells with wild-type B2F1 (at 37°C) no epithelial damage was apparent. Thus, STEC hemolysin produced, regulated, and delivered from wild-type B2F1 does not kill T84 cells *in vitro*, in contrast to the amounts of hemolysin translocated from the bacterium 30.10a. Whether such tight regulation of hemolysin occurs in the infected human host remains to be seen. Some STEC hemolysin is produced in humans as assessed by the documented antibody response of 19 of the 20 serum samples from patients with 0157-associated HUS that reacted with STEC hemolysin (Schmidt et al., 1995).

What role this hemolysin plays in the pathogenesis of STEC remains to be determined. The related α -hemolysin produced and secreted by uropathogenic *E. coli* is known to have a wide spectrum of cytotoxic activity for erythrocytes, granulocytes (Bhakdi et al., 1989; Gadegerg et al., 1984), monocytes (Bhakdi et al., 1990), endothelial cells (Suttorp et al., 1990) and renal

epithelial cells of mice, ruminants, and primates (Keane et al., 1987; Mobley et al., 1990). STEC hemolysin lyses sheep and human erythrocytes and kills bovine leukocytes (albeit with a specific activity more than 20-fold lower than that of α -hemolysin), but unlike α -hemolysin, STEC hemolysin has virtually no activity against human leukocytes (Bauer et al., 1996). Thus, STEC hemolysin may produce non-cytocidal effects on specific human target cells, that have not yet been defined and may affect normal cell functions in various ways. For instance, it has been reported that when sublytic amounts of α -hemolysin are injected into mice, it initiates a cytokine response similar to that seen *in vitro* and elevates the levels of IL-1 and TNF in serum (May et al., 1996). This ability to stimulate cytokine response may aid in the pathogenesis of uropathogenic *E. coli* by redirecting the immune response or by activation of host cell signal transduction that may be advantageous to the bacteria. Another example is intimin-positive STEC that utilize host cell signal transduction that results in intimate attachment of these bacteria to the host epithelial cell. Whether or not STEC-hemolysin serves to upregulate or downregulate receptors on human epithelial cells is not known, but expression of STEC hemolysin appears to be regulated by H-NS *in vitro*.

H-NS typically serves as a repressor of gene expression for those genes that it regulates. The thermoregulation of *E. coli pap* (pyelonephritis-associated pili) transcription by H-NS is one such system. The expression of Pap pili that facilitate the attachment of *E. coli* to uroepithelial cells is shut off outside the host at temperatures below 26°C; but these pili are expressed at 37°C and play a critical role in the colonization of host tissues in upper urinary tract infections (Welch et al., 1981; Hagberg et al., 1983; Lindberg et al., 1984). This type of H-NS-controlled thermoregulatory response has been shown to affect virulence factor expression in many bacterial

genera including *Escherichia* (Roosendaal et al., 1986; Jordi et al., 1992) and *Shigella* (Maurelli et al., 1984). Whether adherence factor(s) expression by B2F1 is thermoregulated by H-NS remains to be determined.

Flagella biogenesis is one of the few examples of a well documented system in which H-NS actually serves as a positive regulator (Bertin et al., 1994; Bertin et al., 1999). Recently, a LEE-encoded regulator (Ler) was localized to *orf1* on the LEE pathogenicity island (Mellies et al., 1999). Ler is a positive regulator that controls the expression of genes of the EPEC LEE pathogenicity island. Ler shares amino acid homology with the DNA structural protein and transcriptional regulator H-NS (24% identity and 44% similarity to H-NS of *Salmonella*; McDaniel, 1986; Elliot et al., 1998; Mees et al., 1999). Ler is also present in *E. coli* 0157:H7 and is an important transcriptional activator of 0157:H7 virulence genes. Intimin-negative STEC do not encode the LEE pathogenicity island or the Ler gene but H-NS encoded by B2F1 may coordinately activate STEC adherence and motility but repress the amount of STEC hemolysin produced. This latter finding, taken with the global virulence regulator effects of the 0157:H7 H-NS-related Ler, suggest that H-NS or H-NS-like molecules may be important as global regulators of STEC in a manner similar to Per (which is not in *E. coli* 0157:H7) and Ler control of EPEC genes of the LEE pathogenicity island.

Whether H-NS is first in the hierarchy of a regulatory cascade for B2F1 or if it is the only global regulator involved in B2F1 adherence remains unknown. Because the structural gene (s) or regulatory genes (except H-NS) involved in adherence were not identified we could not determine if regulation by H-NS occurred by promoter-DNA binding or via protein-protein contact. STEC hemolysin is not a proven virulence factor of STEC bacteria and regulation of

its expression in STEC or 0157:H7 has not been previously described. Coordinated regulation of hemolysin activity and adherence in STEC suggests (but does not prove) that hemolysin plays a role in virulence. Furthermore, regulation of genes encoded by the large plasmid by chromosomal gene products (i.e. H-NS) has not previously been reported in STEC.

Future experiments that attempt to isolate the structural gene involved in adherence should be done in hemolysin-negative derivative S11. To avoid isolation of mutations that de-repress or activate the levels of endogenous alkaline phosphatase, an alkaline phosphatase null mutant should be generated if *Tn_{phoA}* mutagenesis is used. Other translational fusion systems are available to generate protein-fusions to outer membrane, periplasmic or secreted proteins and may be considered in lieu of *Tn_{phoA}* (i.e. beta-lactamase). Alternatively, a transcriptional luciferase-operon-fusion-cosmid bank (or chloramphenicol acetyltransferase system) could be prepared for both mutant 34.7 and wild-type B2F1. A comparison of the genes activated by H-NS in both backgrounds could be made and those that are missing in 34.7 could be recovered from wild-type and studied further. Fortunately, H-NS appears to activate expression of adherence factors in B2F1 and analysis of SDS-PAGE gel protein profiles may show the diminution of or absence of a protein in mutant 34.7 that is present in wild-type. Reverse genetics (though cumbersome and risky) could be used to clone the gene and determine if it is important in adherence. Differential display of prokaryotic messenger RNAs, pioneered by Liang and Pardee in 1992 for use in eukaryotic systems, could be exploited to isolate genes not present in non-pathogenic *E. coli*, that are present in STEC and that are expressed during adherence to T84 cells. Because the *E. coli* K-12 strain DH5 α is poorly adherent to T84 cells, gene transfer of cosmid bank gene clusters from wild type STEC into DH5 α could identify the STEC genes

that encode the adhesin (s). The exact mechanism by which H-NS regulates STEC hemolysin may itself be important to explore and may lead to discovery of an intermediate regulatory gene important in coordinate regulation of the expression of hemolysin and adherence factor(s).

Bibliography

Akerley, B. J., P. A. Cotter, and J. F. Miller. 1995. Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. *Cell* **80**:611-620.

Akerley, B. J., and J. F. Miller. 1993. The *bvgAS* locus negatively controls motility and flagella synthesis in *Bordetella bronchiseptica*. *J. Bacteriol.* **174**:980-990.

Albert, M. J., S. M. Faruque, M. Ansaruzzaman, M. M. Islam, K. Haider, K. Alam, I. Kabir, and R. Robins-Browne. 1992. Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. *J. Med. Microbiol.* **37**:310-314.

Allerberger, F., D. Rossboth, M. P. Dierich, S. Aleksic, H. Schmidt, and H. Karch. 1996. Prevalence and clinical manifestations of Shiga toxin-producing *Escherichia coli* infections in Austrian children. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**:545-550.

Arnqvist, A., A. Olsen, and S. Normark. 1994. σ^S -dependent growth-phase induction of the *csgBA* promoter in *Escherichia coli* can be achieved in vivo by σ^{70} in the absence of the nucleoid-associated protein H-NS. *Mol. Microbiol.* **13**:1021-1032.

Ashkenazi, S., L. May, M. LaRocco, E. L. Lopez, and T. G. Cleary. 1991. The effect of postnatal age on the adherence of enterohemorrhagic *Escherichia coli* to rabbit intestinal cells. *Pediatr. Res.* **29**:14-19.

Atlung ,T., and H. Ingmer. 1997. H-NS: a modulator of environmentally regulated gene expression. *Mol. Microbiol.* **24**:7-17.

Ausbel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith. (ed.) 1990. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.

Baldini, M. M., J. B. Kaper, M. M. Levine, D. C. Candy, and H. W. Moon. 1983. Plasmid-mediated adhesion in enteropathogenic *Escherichia coli*. *J. Pediatr. Gastroenterol. Nutr.* **2**:534-538.

Banatvala, N., M. Debeukelaer, P. Griffin, T. Barrett, K. Greene, J. Green and J. Wells. 1996. Shiga-like toxin-producing *Escherichia coli* 0111 and associated hemolytic-uremic syndrome: a family outbreak. *Pediatr. Infect. Dis. J.* **15**:1008-1011.

Barth, M., C. Marschall, A. Muffler, D. Fischer, and R. Henggeaaronis. 1995. Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of σ^s and many σ^s dependent genes in *Escherichia coli*. *J. Bacteriol.* **177**:3455-3464.

Bauer, M., and R. A. Welch. 1996. Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* 0157:H7. *Infect. Immun.* **64**:167-175.

Benjelloun-Touimi, Z., P. J. Sansonetti, and C. Parsot. 1995. SepA, the major extracellular protein of *Shigella flexneri*: autonomous secretion and involvement in tissue invasion. *Mol. Microbiol.* **17**:123-135.

Benz, I., and M. A. Schmidt. 1989. Cloning and expression of an adhesion (AIDA-I) involved in diffuse adherence of enteropathogenic *Escherichia coli*. *Infect. Immun.* **57**:1506-1511.

Bertin, P., E. Terao, E. H. Lee, P. Lejeune, C. Colson, A. Danchin, and E. Collatz. 1994. The H-NS protein is involved in the biogenesis of flagella in *Escherichia coli*. *J. Bacteriol.* **176**:5537-5540.

Bertin, P., P. Lejeune, C. Laurent-Winther, and A. Danchin. 1990. Mutations in *bglY*, the structural gene for the DNA-binding protein H1, affect expression of several *Escherichia coli* genes. *Biochimie* **72**:889-891.

Bertin, P., N. Benhabiles, E. Krin, C. Laurent-Winter, C. Tendeng, E. Turlin, A. Thomas, A. Danchin and R. Brasseur. 1999. The structural and functional organization

of H-NS-like proteins is evolutionarily conserved in Gram-negative bacteria. *Mol. Microbiol.* **31**:319-329.

Beutin, L., M. A. Montenegro, I. Ørskov, F. Ørskov, J. Prada, S. Zimmermann, and R. Stephan. 1989. Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. *J. Clin. Microbiol.* **27**:2559-2564.

Bhakdi, S., S. Greulich, M. Muhly, B. Eberspacher, H. Becker, a. Thiele, and F. Hugo. 1989. Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. *J. Exp. Med.* **169**:737-754.

Bhakdi, S., M. Muhly, S. Korom, and G. Schmidt. 1990. Effects of *Escherichia coli* hemolysin on human monocytes: cytotoxic action and stimulation of interleukin 1 release. *J. Clin. Invest.* **85**:1746-1753.

Bilge, S. S., C. R. Clausen, W. Lau, and S. L. Moseley. 1989. Molecular characterization of a fimbrial adhesin. F1845, mediating diffuse adherence of diarrhea-associated *Escherichia coli* to HEp-2. *J. Bacteriol.* **171**:4281-4289.

Birnboim, H. C., and J. A. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-1523.

Blattner, F. R., G. I. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277**:1453-1462.

Bokete, T. N., T. S. Whittam, R. A. Wilson, C. R. Clausen, C. M. O'Callahan, S. L. Moseley, T. R. Fritsche, and P. I. Tarr. 1997. Genetic and phenotypic analysis of *Escherichia coli* with enteropathogenic characteristics isolated from Seattle children. *J. Infect. Dis.* **175**:1382-1389.

Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**:85-113.

Bonnet, H., B. Souweine, G. Gauthier, C. Rich, V. Livrelli, J. Sirot, B. Joly, and C. Forestier. 1998. Non-0157:H7 Stx2-producing *Escherichia coli* strains associated with sporadic cases of hemolytic-uremic syndrome in adults. *J. Clin. Microbiol.* **36**:1777-1780.

Bopp, C. A., K. D. Greene, F. P. Downes, E. G. Sowers, J. G. Wells, and I. K. Wachsmuth. 1987. Unusual Verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis. *J. Clin. Microbiol.* **25**:1486-1489.

Bray, J. 1945. Isolation of antigenically homogeneous strains of *Bact. coli Neapolitanum* from summer diarrhoea of infants. *J. Pathol. Bacteriol.* **57**:239-247.

Brewster, D. H., M. I. Brown, D. robertson, G. L. Houghton, J. Bimson, and J. C. Sharp. 1994. An outbreak of *Escherichia coli* 0157 associated with a children's paddling pool. *Epidemiol. Infect.* **112**:441-447.

Brinton, C. C. 1959. Non-flagellar appendages of bacteria. *Nature* **183**:782-786.

Brunder, W., H. Schmidt, and H. Karch. 1996. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohemorrhagic *Escherichia coli* 0157:H7. *Microb.* **142**:3305-3315.

Brunder, W., H. Schmidt, and H. Karch. 1997. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* 0157:H7 cleaves human coagulation factor V. *Mol. Microbiol.* **24**:767-778.

Cantey, J. R., and R. K. Blake. 1977. Diarrhea due to *Escherichia coli* in the rabbit: a novel mechanism. *J. Infect. Dis.* **135**:454-462.

Chapman, P. A., C. A. Siddons, P.M. Zadik, and L. Jewes. 1991. An improved selective medium for the isolation of *Escherichia coli* 0157. *J. Med. Microbiol.* **35**:107-110.

Chomczynski, P. 1992. One-hour alkaline capillary transfer for blotting of DNA and RNA. *Anal. Biochem.* **201**:134-139.

Church, G. M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* **81**:1991-1995.

Ciacchi-Woolwine, F., I. C. Blomfield, S. H. Richardson, and S. B. Mizel. 1998. *Salmonella* flagellin induces tumor necrosis factor alpha in a human promonocytic cell line. *Infect. Immun.* **66**:1127-1134.

Cimolai, N., S. Basalyga, D. G. Mah, B. J. Morrison, and J. E. Carter. 1993. A continuing assessment of risk factors for the development of *Escherichia coli* 0157:H7-associated hemolytic-uremic syndrome. *Clin. Nephrol.* **42**:85-89.

Cimolai, N., B. J. Morrison, and J. E. Carter. 1992. Risk factors for the central nervous system manifestations of gastroenteritis-associated hemolytic uremic syndrome. *Pediatrics* **90**:616-621.

Clark, J. M. 1988. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res.* **16**:9677-9686.

Colonna, B., M. Casalino, P. A. Fradiani, C. Zagaglia, S. Naitza, L. Leoni, G. Prosseda, A. Coppo, P. Ghetardini, and M. Nicoletti. 1995. H-NS regulation of virulence gene expression in enteroinvasive *Escherichia coli* harboring the virulence plasmid integrated into the host chromosome. *J. Bacteriol.* **177**:4703-4712.

Connel, H., W. Agace, P. Klemm, M. Schembri, S. Marild, and C. Svanborg. 1996. Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. *Proc. Natl. Acad. Sci. USA* **93**:9827-9832.

Cotter, P. A., and J. F. Miller. 1994. BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. *Infect. Immun.* **62**:3381-3390.

Cotter, P. A., and Miller, J. F. 1997. A mutation in the *Bordetella bronchiseptica* *bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. *Mol. Microbiol.* **24**:673-687.

Cransberg, K., J. H. Van DenKerkhof, J. R. Bänffer, C. Stijnen, K. Wernars, N. C. Van De Kar, J. Nauta, and E. D. Wolff. 1996. Four cases of hemolytic uremic syndrome-source contaminated swimming water? **46**:45-49.

Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe. 1979. An adhesive factor found in strains of *E. coli* belonging to the traditional infantile enteropathogenic serotypes Curr. Microbiol. **3**:95-99.

Dagberg, B., and B. E. Uhlin. 1992. Regulation of virulence-associated plasmid genes in enteroinvasive *Escherichia coli*. J. Bacteriol. **174**:7606-7612.

DeFez, R., and M. Defelice. 1981. Cryptic operon for β -glucosidase metabolism in *Escherichia coli* K-12: genetic evidence for a regulatory protein. Genetics **97**:11-25.

Deibel, C., S. Krämer, T. Chakraborty, and F. Ebel. 1998. EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells where it appears as a tyrosine-phosphorylated 90 kDa protein. Mol. Microbiol., **28**:463-474.

DeLorenzo, V., M. Herrero, U. Jakubzik, and K. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative Eubacteria. J. Bacteriol. **172**:6568-6572.

Devinney, R., M. Stein, D. Reinscheid, A. Abe, S. Ruschkowski, and B. B. Finlay. 1999. Enterohemorrhagic *Escherichia coli* 0157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. Infect. Immun. **67**:2389-98.

Dharmasathaphorn, K., and J. L. Madara. 1990. Established intestinal cell lines as model systems for electrolyte transport studies. *Methods Enzymol.* **192**:354-389.

Donato, G. M., and T. H. Kawula. 1999. Phenotypic analysis of random *hns* mutations differentiate DNA-binding activity from properties of *fimA* promoter inversion modulation and bacterial motility. *J. Bacteriol.* **181**:941-948.

Donato, G. M., M. J. Lelivelt, and T. H. Kawula. 1997. Promoter-specific repression of *fimB* expression by the *Escherichia coli* nucleoid-associated protein H-NS. *J. Bacteriol.* **179**:6618-6625.

Donnenberg, M. S., J. A. Girón, J. P. Nataro, and J. B. Kaper. 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. *Mol. Microbiol.* **6**:3427-3437.

Donohue-Rolfe, A., M. Jacewicz, and G. T. Keusch. 1989. Isolation and characterization of functional Shiga toxin subunits and renatured holotoxins. *Mol. Microbiol.* **3**:1231-1236.

Donohue-Rolfe, A., G. T. Keusch, C. Edson, D. Thorley-Lawson, and M. Jacewicz. 1984. Pathogenesis of *Shigella* diarrhea. IX. Simplified high yield purification of *Shigella* toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. *J. Exp. Med.* **160**:1767-1781.

Dorman, C. J., J. C. Hinton, and A. Free. 1999. Domain organization and oligomerization among H-NS-like nucleoid-associated proteins in bacteria. *Trends Microbiol.* **7**:124-128.

Dower, W., W. Miller, and C. Ragsdale. 1988. High efficiency transformation of *E. coli* by electroporation. *Nucleic Acids Res.* **16**:6127-6145.

Drolet, R. J. J. M. Fairbrother, J. Harel, and P. Hélie. 1994. Attaching and effacing and enterotoxigenic *Escherichia coli* associated with enteric colibacillosis in the dog. *Can. J. Vet. Res.* **58**:87-92.

Dupont, H. L., S. B. Formal, R. B. Hornick, M. J. Snyder, J. P Libonati, D. G. Sheahan, E. H. LaBrec, and J. P. Kalas. 1971. Pathogenesis of *Escherichia coli* diarrhea. *N. Eng. J. Med.* **285**:1-9.

Durno, C. R., R. Soni, and P. Sherman. 1989. Adherence of vero cytotoxin-producing *Escherichia coli* serotype 0157:H7 to isolated epithelial cells and brush border membranes *in vitro*: role of type 1 fimbriae (pili) as bacterial adhesin expressed by strain CL-49. *Clin. Invest. Med.* **12**:194-200.

Edwards, A. C., A. R. Melton-Celsa, L. Arbuthnott, J. R. Stinson, C. K. Schmitt, H. C. Wong, and A. D. O'Brien. 1998. Vero cell neutralization and mouse protective efficacy of humanized monoclonal antibodies against *Escherichia coli* toxins Stx1 and Stx2, p. 388-392.

In J. B. Kaper, A. D. O'Brien (ed.), *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, D. C.

Elliott, S. J., L. A. Wainwright, T. McDaniel, B. MacNamara, M. Donnenberg, and J. B. Kaper. 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol. Microbiol.* **28**:1-4.

Elliott, S. J., J. Yu, and J. B. Kaper. 1999. The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* 0157:H7 is unable to confer the attaching and effacing phenotype upon *E. coli* K-12. *Infect. Immun.* 1999 **67**:4260-4263.

Fagan, P. K., M. A. Hornitzky, K. A. Bettelheim, and S. P. Djordjevic. 1999. Detection of Shiga-like toxin (*stx1* and *stx2*), intimin (*eaeA*), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. *Appl. Environ. Microbiol.* **65**:868-72.

Fasano, A., B. A. Kay, R. G. Russell, D. R. Maneval, and M. M. Levine. 1990. Enterotoxin and cytotoxin production by enteroinvasive *Escherichia coli*. *Infect. Immun.* **58**:3717-3723.

Feng, P. K. A. Lampel, H. Karch, and T. S. Whittam. 1998. Genotypic and phenotypic changes in the emergence of *Escherichia coli* 0157:H7. *J. Infect. Dis.* **177**:1750-1753.

Fischer, J. C. Maddox, R. Moxley, D. Kinden, and M. Miller. 1994. Pathogenicity of a bovine attaching effacing *Escherichia coli* isolate lacking Shiga-like toxins. *Am. J. Vet. Res.* **55**:991-999.

Frankel, G., O. Lider, R. HersHKoviz, A. P. Mould, S. G. Kachalsky, D. C. A. Candy, L. Cahalon, M. J. Humphries, and G. Dougan. 1996. The cell-binding domain of intimins from enteropathogenic *Escherichia coli* binds to β_1 integrins. *J. Biol. Chem.* **271**:20359-20364.

Fratamico, P. M., S. Bhaduri, and R. L. Buchanan. 1993. Studies on *Escherichia coli* serotype 0157:H7 strains containing a60-MDa plasmid and on 60-MDa plasmid-cured derivatives. *J. Med. Microbiol.* **39**:371-381.

Free, A., and C. J. Dorman. 1995. Coupling of *Escherichia coli* hns mRNA levels to DNA synthesis by autoregulation: implications for growth-phase control. *Mol. Microbiol.* **18**:101-113.

Gadeberg, O. V., and I. Ørskov. 1984. In vitro cytotoxic effect of α -hemolytic *Escherichia coli* on human blood granulocytes. *Infect. Immun.* **45**:255-260.

Gentry, M. K., and J. M. Dalrymple. 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. *J. clin. Microbiol.* **12**:361-366.

Girón, J. A., A. S. Ho, and G. K. Schoolnik. 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science* **254**:710-713.

Girón, J. A., M. M. Levine, and J. B. Kaper. 1994. Longus: along pilus ultrastructure produced by human enterotoxigenic *Escherichia coli*. *Mol. Microbiol.* **12**:71-82.

Girón, J. A., A. S. Ho, and G. K. Schoolnik. 1993. Characterization of fimbriae produced by enteropathogenic *Escherichia coli*. *J. Bacteriol.* **175**:7391-7403.

Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann, and A. Danchin. 1988. Secretion of cytolysin, the calmodulin sensitive adenylate cyclase-hemolysin bifunctional protein of *Bordetella pertussis*. *EMBO J.* **7**:3997-4004.

Göransson, M., B. Sonden, P. Nilsson, B. Dagberg, D. Forsman, K. Emanuelsson, and B. E. Uhlin. 1990. Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. *Nature (London)*. **344**:682-685.

Grant, S. G., J. J. Bloom, and D. Hanahan. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc. Natl. Acad. USA* **12**:4645-4649.

Griffin, P.M., S.M. Ostroff, R.V Tauxe, K.D Greene, J.G. Wells, J.H. Lewis, and P.A. Blake. 1988. Illnesses associated with *Escherichia coli* 0157:H7 infections. *Ann. Intern. Med.* **109**:705-712.

Griffin, P. M. 1995. *Escherichia coli* 0157:H7 and other enterohemorrhagic *Escherichia coli*, p. 739-761. In M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), *Infections of the gastrointestinal tract*. Center for Disease Control. U. S. Department of Health and Human Services.

Gross, R. J., S. M. Scotland, and B. Rowe. 1976. Enterotoxin testing of *Escherichia coli* causing epidemic infantile enteritis in the U. K. *Lancet* **1**:629-631.

Gunzer, F., H. Bohm, H.Russmann, M. Bitzan, S. Aleksic, and H. Karch. 1992. Molecular detection of sorbitol-fermenting *Escherichia coli* 0157 in patients with hemolytic-uremic syndrome. *J. Clin. Microbiol.* **30**:1807-1810.

Gunzer, F., and H. Karch. 1983. Expression of A and B subunits of Shiga-like toxin II as fusions with glutathione s-transferase and their potential for use in seroepidemiology. *J. Clin. Microbiol.* **31**:2604-2610.

Gyles, C., R. Johnson, A. Gao, K. Ziebell, D. Pierard, S. Aleksie, and P. Boerlin. 1998. Association of enterohemorrhagic *Escherichia coli* hemolysin with serotypes of Shiga-like-toxin-producing *Escherichia coli* of human and bovine origins. *Appl. Environ. Microbiol.* **64**:4134-4141.

Hagberg, L., R. S. Hull, S. Falkow, R. Freter, and C. Svanborg-Eden. 1983. Contribution of adhesion and bacterial persistence in the mouse urinary tract. *Infect. Immun.* **40**:265-272.

Hale, T. L., E. V. Oaks, and S. B. Formal. 1985. Identification and antigenic characterization of virulence-associated, plasmid-coded proteins of *Shigella* spp. and enteroinvasive *Escherichia coli*. *Infect. Immun.* **50**:620-629.

Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner. 1989. New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol.* **171**:4617-4622.

Harrison, J. A., D. Pickard, C. F. Higgins, A. Khan, S. N. Chatfield, T. Ali, C. J. Dorman, C. E. Hormaeche, and G. Dougan. 1994. Role of *hns* in the virulence phenotype of pathogenic salmonellae. *Mol. Microbiol.* **13**:133-140.

Hayes, P. S., K. Blom, P. Feng, J. Lewis, N. A. Strockbine, and B. Swaminathan. 1995. Isolation and characterization of a β -D-glucuronidase-producing strain of *Escherichia coli* serotype 0157:H7 in the United States. *J. Clin. Microbiol.* **33**:3347-3348.

Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, L. R. Booth, G. May, and E. Bremer. 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E.coli*. *Cell* **52**:569-584.

Hinton, J. C., D. S. Santos, A. Seirafi, C. S. Hulton, G. D. Pavitt, and C. F. Higgins. 1992. Expression and mutational analysis of the nucleoid-associated protein H-NS of *Salmonella typhimurium*. *Mol. Microbiol.* **6**:2327-2337.

Ito, H., A. Terai, H. Kurazono, Y. Takeda, and M. Nishibuchi. 1990. Cloning and nucleotide sequencing of Vero toxin 2 variant genes from *Escherichia coli* 091:H21 isolated from a patient with the hemolytic uremic syndrome. *Microbiol. Pathog.* **8**: 47-60.

Ismaili, A., D. Philpott, D. M. McKay, M. H. Perdue, and P. M. Sherman. 1998. Epithelial cell responses to Shiga toxin-producing *Escherichia coli* infection, p.213-225. *In*

J. B. Kaper, A. D. O'Brien (ed.), *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, D. C.

Izumiya, H., J. Terajima, A. Wasa, Y. Inagaki, K. Itoh, K. Tamura, and H. Watanabe. 1977. Molecular typing of *Escherichia coli* 0157:H7 isolates in Japan by using pulsed-field gel electrophoresis. *J. Clin. Microbiol.* **35**:1675-1680.

Jacewicz, M. S., D. W. Acheson, D. G. Binion, G. A. West, L. L. Lincicome, C. Fiocchi, and G. T. Keusch. 1999. Responses of human intestinal microvascular endothelial cells to Shiga toxins 1 and 2 and pathogenesis of hemorrhagic colitis. *Infect. Immun.* **67**:1439-44.

Jarvis, K. G., and J. B. Kaper. 1996. Secretion of extracellular proteins by enterohemorrhagic *Escherichia coli* via a putative type III secretion system. *Infect. Immun.* **64**:4826-4829.

Jarvis, K. G., J. a. Girón, A. E. Jerse, T. K. McDaniel, M. S. Sonnenberg, and J. B. Kaper. 1995. Enteropathogenic *Escherichia coli* contains a specialized secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proc. Natl. Acad. Sci. USA* **92**:7996-8000.

Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper. 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc. Natl. Acad. Sci. USA* **86**:7839-7843.

Johnson, R. P., R. C. Clarke, J. B. Wilson, S. Read, K. Rahn, s. Renwick, K. Sandhu, D. Alves, M. Karmali, H. Lior, S. McEwen, J. Spika, and C. Gyles. 1996. Growing concerns and recent outbreaks involving non-0157:H7 serotypes of verotoxigenic *Escherichia coli*. *J. Food Prot.* **59**:1112-1122.

Jordi, B. J., B. Dagberg, L. A. deHaan, A. M. Hamers, B. A. van der Zeijst, W. Gastra, and B. E. Uhlin. 1992. The positive regulator CfaD overcomes the repression mediated by histone-like protein H-NS (H1) in the CFA/1 fimbrial operon of *Escherichia coli*. *EMBO J.* **11**:2627-2632.

Kado, C. I., and S. T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145**:1365-1373.

Kaper, J. B., Gansheroff, L. J., M. R. Wachtel, and Alison D. O'Brien. 1998. Intimin-mediated adherence of Shiga toxin-producing *Escherichia coli* and attaching-and-effacing pathogens, p. 148-156. *In* J. B. Kaper, A. D. O'Brien (ed.), *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E.coli* strains. ASM Press, Washington, D. C.

Karch, H., J. Heesemann, R. Laufs, A. D. O'Brien, C. O. Tacket, and M. M. Levine.

1987. A plasmid of enterohemorrhagic *Escherichia coli* 0157:H7 is required for expression of a new fimbrial antigen for adhesion to epithelial cells. *Infect. Immun.* **55**:455-461.

Karch, H., N. Stockbine, and A. O'Brien. 1986. Growth of *Escherichia coli* in the presence of trimethoprim-sulfamethoxazole facilitates detection of Shiga-like toxin producing strains by colony blot assay. *FEMS Microbiol. Lett.* **35**:141-145.

Karch, H., H. Schmidt, and W. Brunder. 1998. Plasmid-encoded determinants of *Escherichia coli* 0157:H7, p. 183-194. In J. B. Kaper, and A. D. O'Brien (ed.), *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E.coli* strains. ASM Press, Washington, D. C.

Karmali, M. A., B. T. Steele, M. Petric and C. Lim. 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* **i**:619-620.

Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior. 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. *J. Infect. Dis.* **151**:775-782.

Kawula, T. H., and P. E. Orndorff. 1991. Rapid site-specific DNA inversion in *Escherichia coli* mutants lacking the histonelike protein H-NS. *J. Bacteriol.* **173**:4116-4123.

Keane, W. F., R. A. Welch, G. Gekker, and P. K. Peterson. 1987. Mechanism of *Escherichia coli* α -hemolysin induced injury to isolated renal tubular cells. *Am. J. Pathol.* **126**:350-357.

Keith, B. R., P. A. Maurer, P. A. Spears, and P. E. Orndorff. 1986. Receptor-binding function of type 1 pili affects bladder colonization by a clinical isolate of *Escherichia coli*. *Infect. Immun.* **53**:693-696.

Kenny, B. 1999. Phosphorylation of tyrosine 474 of the enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. *Mol. Microbiol.* **31**:1229-1241.

Kenny, B., and B. B. Finlay. 1997. Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C γ 1. *Infect. Immun.* **65**:2528-2536.

Kenny, B., and B. B. Finlay. 1995. Protein secretion by enteropathogenic *Escherichia coli* is essential for transducing signals to epithelial cells. *Proc Natl. Acad. Sci. USA* **92**:7991-7995.

Kidd, V. J. 1983. In-situ hybridization to agarose gels. Bethesda Research Laboratories Focus **6**:3-4.

Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish. 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **57**:1290-1298.

Kolter, R., M. Inuzuka, and D. R. Helinski. 1978. Transcomplementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. *Cell* **15**:1199-1208.

Konowalchuk J., J. L. Speir, and S. Stavric. 1977. Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun.* **18**:775-779.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* **227**:680-685.

Laurent-Winter, C., S. Ngo, A. Danchin, and P. Bertin. 1997. Role of *Escherichia coli* histone-like nucleoid-structuring protein in bacterial metabolism and stress response-identification of targets by two-dimensional electrophoresis. *Eur. J. Biochem.* **244**:767-773.

Laurent-Winter, C., P. Lejeune, and A. Danchin. 1995. The *Escherichia coli* DNA-binding protein H-NS is one of the first proteins to be synthesized after a nutritional upshift. *Res. Microbiol.* **146**:5-16.

Levine, M. M. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic,

enteroinvasive, enterohemorrhagic, and enteroadherent. *J. Infect. Dis.* **155**:377-389.

Levine, M. M., E. J. Bergquist, D. R., D. H. Waterman, R. B. Hornick, C. R. Young, S. Sotman, and B. Rowe. 1978. *Escherichia coli* strains that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet* **i**:1119-1122.

Levine, M. M., J. Xu, J. B. Kaper, H. Lior, V. Prado, B. Tall, J. Nataro, H. Karch, and K. Wachsmuth. 1987. A DNA probe to identify enterohemorrhagic *Escherichia coli* of 0157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. *J. Infect. Dis.* **156**:175-182.

Liang, P., and A. B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**:967-973.

Lindberg, F. P., B. Lund, and S. Normark. 1984. Genes of pyelonephritogenic *E.coli* required for digalactosidase-specific agglutination of human cells. *EMBO J.* **3**:1167-1173.

Lindgren, S. W., A. R. Melton-Celsa, and A. D. O'Brien. 1993. Virulence of enterohemorrhagic *Escherichia coli* 091:H21 clinical isolates in an orally infected mouse model. *Infect. Immun.* **61**:3832-3842.

Lior, H. 1994. *Escherichia coli* 0157:H7 and verotoxigenic *Escherichia coli* (VTEC). Dairy Food Environ. Sanit. **14**:378-382.

Louise, C. B., and T. G. Obrig. 1995. Specific interaction of *Escherichia coli* 0157:H7-derived Shiga-like toxin II with human renal endothelial cells. J. Infect. Dis. **172**:1397-1401.

Lucht, J. M., P. Dersch, B. Kempf, and E. Bremer. 1994. Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. J. Biol. Chem. **269**:6578-6586.

Madara, J. L., and K. Dharmasathaphorn. 1985. Occluding junction structure-function relationships in a cultured monolayer. J. Cell Biol. **101**:2124-2133.

Makino, K., K. Ishii, T. Yasunaga, M. Hattori, K. Yokoyama, C. H. Yutsudo, Y. Kubota, Y. Yamaichi, T. Iida, K. Yamamoto, T. Honda, C. Han, E. Ohtsubo, M. Kasamatsu, T. Hayashi, S. Kuhara, and H. Shinagawa. 1998. Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic *Escherichia coli* 0157:H7 derived from Sakai outbreak. DNA Res. **5**:1-5.

Maloney, P. C. H. Schneider, and B. L. Brandt. 1972. Production and degradation of serogroup B *Neisseria meningitidis* polysaccharide. Infect. Immun. **6**:657-661.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

March, S. B., and S. Ratnam. 1986: Sorbitol-MacConkey medium for detection of *Escherichia coli* 0157:H7 associated with hemorrhagic colitits. J. Clin. Microbiol. **23**:869-872.

Mathewson, J. J., R. A. Oberhelman, H. L. Dupont, F. Javier de la Cabaca, and E. V. Garibay. 1987. Enteroadherent *Escherichia coli* as a cause of diarrhea among children in Mexico. J. Clin. Microbiol. **25**:1913-1919.

Maurelli, A. T., and P. J. Sansonetti. 1988. Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. Proc. Natl. Acad. Sci. USA **85**:2820-2824.

May, G., P. Dersch, M. Haardt, A. Middendorf, and E. Bremer. 1990. The *osmZ* (*bglY*) gene encodes the DNA-binding protein H-NS (H1a), a component of the *Escherichia coli* K12 nucleoid. Mol. Gen. Genet. **224**:81-90.

May, A. K., R. G. Sawyer, T. Gleason, A. Whitworth, and T. L. Pruett. 1996. In vivo cytokine response to *Escherichia coli* alpha-hemolysin determined with genetically

engineered hemolytic and nonhemolytic *Escherichia coli* variants. *Infect. Immun.* **64**:2167-2171.

McCormick, B. A., D. P. Franklin, D. C. Laux, and P. S. Cohen. 1989. Type 1 pili are not necessary for colonization of the streptomycin-treated mouse large intestine by type-1 pilated *Escherichia coli* F-18 and *E.coli* K-12. *Infect. Immun.* **57**:3022-3029.

McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc. Natl. Acad. Sci. USA* **92**:1664-1668.

McDaniel, T. K., and J. B. Kaper. 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E.coli* K-12. *Mol. Microbiol.* **23**:399-407.

McKee , M.L., A. R. Melton-Celsa, R. A. Moxley, D. H. Francis, and A. D. O'Brien. 1995. Enterohemorrhagic *Escherichia coli* 0157:H7 requires intimin to colonize the gnotobiotic pig intestine and to adhere to HEp-2 cells. *Infect. Immun* **63**:2225-2233.

Mead, D. A., N. J. Pey, C. Hernstadt, R. A. Marcil, and L. M. Smith. 1991. A universal method for the direct cloning of PCR amplified nucleic acid. *Bio/Technology* **9**:657-663.

Mellies, J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper. 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Mol. Microbiol.* **32**:296-306.

Mendel, M., and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. *J. Mol. Biol.* **53**:159-162.

Meng J., S. Zhao, M. P. Doyle. 1998. Virulence genes of Shiga toxin-producing *Escherichia coli* isolated from food, animals and humans. *Int. J. Food Microbiol.* **22**:229-35.

Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575-2583.

Mobley, H. L., D. M. Green, A. L. Trifillis, D. E. Johnson, G. R. Chppendale, C. V. Lockatell, B. D. Jones, and J. W. Warren. 1990. Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. *Infect. Immun.* **58**:1281-1289.

- Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect. Immun.* **41**:1340-1351.
- Nataro, J., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **11**:142-210.
- Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine.** 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEP-2 cells and hemagglutination of human erythrocytes. *Infect. Immun.* **60**:2297-2304.
- Newland, J. W., N. A. Strockbine, S. F. Miller, A. D. O'Brien, and R. K. Holmes.** 1985. Cloning of Shiga-like toxin structural genes from a toxin converting phage of *Escherichia coli*. *Science* **230**:179-181.
- Ofek, I., D. Mirelman, and N. Sharon.** 1977. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature (London)* **265**:623-625.
- Oshima, T., K. Ito, H. Kabayama, and Y. Nakamura.** 1995. Regulation of *lrp* gene expression by H-NS and Lrp proteins in *Escherichia coli*: dominant negative mutations in *lrp*. *Mol. Gen. Genet.* **247**:521-528.

Ostroff, S. M., J. M. Kobayashi, and J. H. Lewis. 1989. Infections with *Escherichia coli* 0157:H7 in Washington State; the first year of statewide disease surveillance, *JAMA* **262**:355-359.

O'Brien, A. D., and G. D. LaVeck, M. R. Thompson, and S. B. Formal. 1982. Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. *J. Infect. Dis.* **146**:763-769.

O'Brien, A. D., T. a. Lively, M. Chen, S. W. Rothman, and S. B. Formal. 1983. *Escherichia coli* 0157:H7 strains associated with haemorrhagic colitis in the United States produce a *Shigella dysenteriae* 1 (Shiga) like cytotoxin. *Lancet* **i**:702.

O'Brien, A. d., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. t. Keusch. 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis, p. 66-94. *In* P. J. Sansonetti (ed.), *Pathogenesis of Shigellosis*. Springer-Verlag KG, Berlin-Heidelberg, Germany.

O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, and S. B. Formal. 1984. Shiga-like toxin-converting phages from *Escherichia coli*. *Science* **226**:694-696.

O'Brien, A. D., and G. D. LaVeck. 1983. Purification and characterization of a *shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. *Infect. Immun.* **40**:675-683.

O'Brien, A. D., M. R. Thompson, J. R. Cantey, and S. B. Formal. 1977. Production of a *Shigella dysenteriae*-like toxin by pathogenic *Escherichia coli*, abstr. B-103, p. 32. In Abstracts of the 77th Annual Meeting of the American Society for Microbiology 1977. American Society for Microbiology, Washington, D. C.

Perera, L. P., L. R. Marques, and A. D. O'Brien. 1988. Isolation and characterization of monoclonal antibodies to Shiga-like toxin II of enterohemorrhagic *Escherichia coli* and use of the monoclonal antibodies in a colony enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **26**:2127-2131.

Perna, N. T., G. F. Mayhew, G. Pósfal, S. J. Elliott, M. S. Donnenberg, J. B. Kaper, and F. R. Blattner. 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* 0157:H7. *Infect. Immun.* **66**:3810-3817.

Philpott, D. J., C. A. Ackerley, a. J. Kiliaan, M. A. Karmali, M. H. Perdue, and P. M. Sherman. 1997. Translocation of verotoxin-1 across T84 monolayers: mechanism of bacterial toxin penetration of epithelium. *Am. J. Physiol.* **273**:G1349-G1358.

Pon, C. L., R. A. Calogero, and C. O. Gualerzi. 1988. Identification, cloning, nucleotide sequence and chromosomal map location of *hns*, the structural gene for *Escherichia coli* DNA-binding protein H-NS. *Mol. Gen. Genet.* **212**:199-202.

Provence, D. L., and R. Curtiss III. 1994. Isolation and characterization of a gene involved in hemagglutination by an avian pathogenic *Escherichia coli* strain. *Infect. Immun.* **62**:1369-1380.

Raleigh, E. A., N. E. Murray, H. Revel, R. M. Blumenthal, D. Westaway, A. D. Reith, P. W. Rigby, J. Elhai, and D. Hanahan. 1988. McrA and McrB restriction phenotypes of some *E. coli* strains and implications for gene cloning. *Nucleic Acids Res.* **16**:1563-1575.

Reilly, A. 1998. Prevention and Control of enterohaemorrhagic *Escherichia coli* (EHEC) infections: Memorandum from a WHO meeting. *Bulletin of the World Health Organization.* **76**:245-255.

Remuzzi, G., and P. Ruggenenti. 1998. The hemolytic uremic syndrome. *Kidney Int.* **53**:54-57.

Renwick, S. A., J. B. Wilson, and R. C. Clarke. 1993. Evidence of direct transmission of *Escherichia coli* 0157:H7 infection between calves and a human. *J. Infect. Dis.* **68**:792-793.

Renz, M., and C. Kurz. 1984. A colorimetric method for DNA hybridization. *Nucleic Acids Res.* **12**:3435-3444.

Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308**:681-685.

Robins-Browne, R. M. 1987. Traditional enteropathogenic *Escherichia coli* of infantile diarrhea. Rev. Infect. Dis. **9**:28-53.

Roosendaal, B., P. M. vanBergehen-Henegouwen, and F. K. deGraff. 1986. Subcellular localization of K99 fimbrial subunits and effect of temperature on subunit synthesis and assembly. J. Bacteriol. **165**:1029-1032.

Rosenshine, I., S. Ruschowski, M. Stein, D. Reinscheid, S. D. Mills, and B. B. Finlay. 1996. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. EMBO J. **15**:2613-2624.

Rothbaum, R., A. J. Mcadams, R. Giannella, and J. C. Partin. 1982. A clinico-pathological study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. Gastroenterology **83**:441-454.

Samadpour, M., L. Grimm, B. Desai, D. Alfi, J. E. Ongerth, and P. I Tarr. 1993. Molecular epidemiology of *Escherichia coli* 0157:H7 strains using bacteriophage λ -restriction

fragment length polymorphism analysis: application to a multistate foodborne outbreak and a day care center cluster. *J. Clin. Microbiol.* **31**:3179-3183.

Sanger, F. S., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.

Savarino, S. J., P. Fox, D. Yikang, and J. P. Nataro. 1994. Identification and characterization of a gene cluster mediating enteroaggregative adherence fimbria 1 biogenesis. *J. Bacteriol.* **176**:4949-4957.

Savarino S. J., A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry. 1993. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E.coli* heat-stable toxin. *Proc. Natl. Acad. Sci. USA* **90**:3093-3097.

Savarino, S. J., A. Fasano, D. C. Robertson, and M. M. Levine. 1991. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an *in vitro* rabbit intestinal model. *J. Clin. Invest.* **87**:1450-1455.

Schaeffer, A. J., W. R. Schwan, S. J. Hultgren, and J. L. Duncan. 1987. Relationship of type 1 pilus expression in *Escherichia coli* to ascending urinary tract infections in mice. *Infect. Immun.* **55**:373-380.

Schauer, D. B., and S. Falkow. 1993. The *eae* gene of *Citrobacter freundii* biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia. *Infect. Immun.* **61**:4654-4661.

Schmidt, H., C. Geitz, P. I. Tarr, M. Frosch, and H. Karch. 1999. Non-0157:H7 pathogenic Shiga toxin-producing *Escherichia coli*: phenotypic and genetic profiling of virulence traits and evidence for clonality. *J. Infect. Dis.* **179**:115-123.

Schmidt, H., L. Beutin, and H. Karch. 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* 0157:H7 strain EDL 933. 1995. *Infect. Immun.* **63**:1055-1061.

Schmidt, H., C. Kernbach, and H. Karch. 1996. Analysis of the EHEC *hly* operon and its location in the physical map of the large plasmid of enterohemorrhagic *Escherichia coli* 0157:H7. *Microbiol.* **142**:907-914.

Schmidt, H., B. Henkel, H. Karch. 1997. A gene cluster closely related to type II secretion pathway operons of Gram-negative bacteria is located on the large plasmid of enterohemorrhagic *Escherichia coli* 0157 strains. *FEMS Microbiol. Lett.* **148**:265-272.

Schmidt, H., H. Rüssmann, and H. Karch. 1993. Virulence determinants in nontoxinogenic *Escherichia coli* 0157 strains that cause infantile diarrhea. *Infect. Immun.* **61**:4894-4898.

Schmidt, H., H. Karch, and L. Beutin. 1994. The large-sized plasmids of enterohemorrhagic *Escherichia coli* 0157 strains encode hemolysins which are presumably members of the *E.coli* alpha-hemolysin family. *FEMS Microbiol. Lett.* **117**:189-196.

Schmitt, C. K., M. L. McKee, and A. D. O'Brien. 1991. Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of 0157:H- strain E32511. *Infect. Immun.* **59**:1065-1073.

Schmitt, C. K., S. C. Darnell, V. L. Tesh, B. A. Stocker, and A.D. O'Brien. 1994. Mutation of *flgM* attenuates virulence of *Salmonella typhimurium*, and mutation of *fliA* represses the attenuated phenotype. *J. Bacteriol.* **176**:368-377.

Schmitt, C. K., S. C. Darnell, and A. D. O'Brien. 1996. The attenuated phenotype of a *Salmonella typhimurium flgM* mutant is related to expression of FliC flagellin. *J. Bacteriol.* **178**:2911-2915.

Schweizer H., and W. Boos. 1983. Transfer of the delta (arg F-lac) U169 mutation between *Escherichia coli* strains by selection for a closely linked Tn10 insertion. *Mol. Gen. Genet.* **192**:293-295.

Scotland, S. M., H. R. Smith, and B. Rowe. 1985. Two distinct toxins active on Vero cells from *Escherichia coli* 0157:H7. *Lancet* **2**:885-886.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. H. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **51**:873-884.

Shi, X., B. C. Waasdorp, and G. N. Bennett. 1993. Modulation of acid-induced amino acid decarboxylase gene expression by *hns* in *Escherichia coli*. *J. Bacteriol.* **175**:1182-1186.

Shindo, H., T. Iwaki, R. Ieda, H. Kurumizaka, C. Ueguchi, T. Mizuno, S. Morikawa, H. Nakamura, and H. Kuboniwa. 1995. Solution structure of the DNA binding domain of a nucleoid-associated protein, H-NS, from *Escherichia coli*. *FEBS Lett.* **360**:125-131.

Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1**:784-791

Slutsker, L., A. Ries, Greene, K., Wells, J., Hutwagner, L., Griffin, P. 1997. *Escherichia coli* O157:H7 diarrhea in the United States: clinical and epidemiologic features. *Ann. Intern. Med.* **126**:505-513.

Sokurenko, E. V., H. S. Courtney, D. E. Ohman, P. Klemm, and D. L. Hasty. 1994. FimH family of type 1 fimbrial adhesins: functional heterogeneity due to minor sequence variations among *fimH* genes. *J. Bacteriol.* **176**:748-755.

Soloaga, A., M. P. Veiga, L. M. Garcia-Segura, H. Ostolaza, R. Brasseur and F. M. Goni. 1999. Insertion of *Escherichia coli* α -hemolysin in lipid bilayers as a non-transmembrane integral protein: prediction and experiment. *Mol. Microbiol.* **31**:1013-1024.

Sondén, B., and B. E. Uhlin. 1996. Coordinated and differential expression of histone-like proteins in *Escherichia coli*: regulation and function of the H-NS analog StpA. *EMBO J* **15**:4970-4980.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.

Spassky, S. Rimsky, H. Garreau, and H. Buc. 1984. H1a, an *E.coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro*. Nucleic Acids Res. **12**:5321-5340.

Spurio, R., M. Falconi, A. Brandi, C. L. Pon, and C. O. Gualerzi. 1997. The oligomeric structure of nucleoid protein H-NS is necessary for recognition of intrinsically curved DNA and for DNA bending. EMBO J. **16**:1795-1805.

Staley, T. E., E. W. Jones, and L. D. Corley. 1969. Attachment and penetration of *Escherichia coli* into the intestinal epithelium of the ileum in newborn pigs. Am. J. Pathol. **56**:371-392.

Stanley, P., V. Koronakis, and C. Hughes. 1998. Acylation of *Escherichia coli* hemolysin: a unique protein lipidation mechanism underlying toxin function. Microbiol. Mol. Biol. Rev. **62**:309-333.

Stein, M., B. Kenny, M. A. Stein , and B. B. Finlay. 1996. Characterization of EspC, a 110-kilodalton protein secreted by enteropathogenic *Escherichia coli* which is homologous to members of the immunoglobulin a protease-like family of secreted proteins. J. Bacteriol. **178**:6546-6554.

Stein, P. E., A. Boodhoo, G. T. Tyrell, J. Brunton, and R. J. Read. 1992. Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E.coli*. *Nature* **355**:748-750.

Strathdee, C. a., and R. Y. Lo. 1987. Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-hemolysin of *Escherichia coli*. *Infect. Immun.* **55**:3222-3236.

Strockbine, N., K. Sowers, K. Greene, P. Hayes, P. Griffin, and J. Wells. 1997. Characterization of Shiga toxin-producing non-0157 *Escherichia coli* from the United States, 1983-1997, abstr. V234/III, p.67. In 3rd International Symposium and Workshop on Shiga toxin (Verocytotoxin)-Producing *Escherichia coli* Infections.

Strockbine, N. A., L. R. Marques, R. K. Holmes, and A. D. O'Brien. 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. *Infect. Immun.* **50**:695-700.

Suttorp, N., M. Fuhrmann, S. Tannertotto, F. Grimminger, and S. Bhakdi. 1993. Pore-forming bacterial toxins potently induce release of nitric oxide in porcine endothelial cells. *J. Exp. Med.* **178**:337-341.

Suzuki, T., C. Ueguchi, and T. Mizuno. 1996. H-NS regulates OmpF expression through micF antisense RNA in *Escherichia coli*. *J. Bacteriol.* **178**:3650-3653.

- Tanaka, K., S. Muramatsu, H. Yamada, and T. Mizuno.** 1991. Systematic characterization of curved DNA segments randomly cloned from *Escherichia coli* and their functional significance. *Mol. Gen. Genet.* **226**:367-376.
- Tarr, P. I.** 1994. *Escherichia coli* 0157:H7: clinical, diagnostic, and epidemiological aspects of human infection. *Clin. Infect. Dis.* **20**:1-10.
- Tesh, V. L., J. A. Burris, J. W. Owens, V. M. Gordon, E. A. Wadolkowski, A. D. O'Brien, and J. E. Samuel.** 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.* **61**:3392-3402.
- Tippner, D., and R. Wagner.** 1995. Fluorescence analysis of the *Escherichia coli* transcription regulator H-NS reveals two distinguishable complexes dependent on binding to specific or nonspecific DNA sites. *J. Biol. Chem.* **270**:22243-22247.
- Tippner, D., H. Afflerbach, C. Bradaczek, and R. Wagner.** 1994. Evidence for a regulatory function of the histone-like *Escherichia coli* protein H-NS in ribosomal RNA synthesis. *Mol. Microbiol.* **11**:589-604.
- Tobe, T., M. Yoshikawa, T. Mizuno, and C. Sasakawa.** 1993. Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by VirF and repression by H-NS. *J. Bacteriol.* **175**:6142-6149.

Toth, I., M. L. Cohen, H. S. Rumschlag, L. W. Riley, E. H. White, J. H. Carr, W. W. Bond, and I. K. Wachsmuth. 1990. Influence of the 60-megadalton plasmid on adherence of *Escherichia coli* 0157:H7 and genetic derivatives. *Infect. Immun.* **58**:1223-1231.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.

Tupper, A. E., T. A. Owen-Hughes, D. W. Ussery, D. S. Santos, D. J. Ferguson, J. M. Sidebotham, J. C. Hinton, and C. F. Higgins. 1994. The chromatin-associated protein H-NS alters DNA topology in vitro. *EMBO J.* **13**:258-268.

Tuttle, J., T. Gomez, M. P. Doyle, J. G. Wells, T. Zhao, R. V. Tauxe, P.M. Griffin. 1999. Lessons from a large outbreak of *Escherichia coli* 0157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol. Infect.* **122**:185-192.

Tzipori, S., R. M. Robins-Browne, G. Gonis, J. Hayes, M. Withers, and E. McCartney. 1985. Enteropathogenic *Escherichia coli* enteritis: evaluation of the gnotobiotic piglet as a model of human infection. *Gut* **26**:570-578.

Tzipori, S., H. Karch, K. I. Wachsmuth, R. M. Robins-Browne, A. D. O'Brien, Hermy Lior, M. L. Cohen, J. Smithers, and M. M. Levine. 1987. Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* 0157:H7 in gnotobiotic piglets. **55**:3117-3125.

Ueguchi, C., T. Suzuki, T. Yoshida, K. Tanaka, and T. Mizuno. 1996. Systematic mutational analysis revealing the functional domain organization of *Escherichia coli* nucleoid protein H-NS. *J. Mol. Biol.* **263**:149-162.

Ueguchi, C., C. Seto, T. Suzuki, and T. Mizuno. 1997. Clarification of the dimerization domain and its functional significance for the *Escherichia coli* nucleoid protein H-NS. *J. Mol. Biol.* **274**:145-151.

Ueguchi, C., and T. Mizuno. 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. *EMBO J.* **12**:1039-1046.

Ulshen, M. H., and J. L. Rollo. 1980. Pathogenesis of *Escherichia coli* gastroenteritis in man: another mechanism. *N. Engl. J. Med.* **302**:99-101.

Ussery, D. W., J. C. D. Hinton, B. J. A. M. Jordi, P. E. Granum, A. Seirafi, R. J. Stephen, a. E. Tupper, G. Berridge, J. M. Sidebotham, and C. F. Higgins. 1994. The chromatin-associated protein H-NS. *Biochimie.* **76**:968-980.

Wadolowski, E. A., L. M. Sung, J. A. Burris, J. E. Samuel, and A. D. O'Brien. 1990.

Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin type II. *Infect. Immun.* **58**:3959-3965.

Wadolowski, E. A., J. A. Burris, and A. D. O'Brien. 1990. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* 0157:H7. *Infect. Immun.* **58**:2438-2445.

Wandersman, C., and P. Delepelaire. 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc. Natl. Acad. Sci. USA* **87**:4776-4780

Weikel, C. S., K. M. Tiemens, S. L. Moseley. 1988. Species specificity and lack of production of STB enterotoxin by *Escherichia coli* strains isolated from humans with diarrheal illness. *Infect. Immun.* **56**:323-325.

Weikel, C. S., H. N. Nellans, and R. L. Guerrant. 1986. In vivo and in vitro effects of a novel enterotoxin, ST_b, produced by *Escherichia coli*. *J. Infect. Dis.* **153**:893-901.

Welch, R. A. 1987. Identification of two different hemolysin determinants in uropathogenic *Proteus* isolates. *Infect. Immun.* **55**:2183-2190.

Welch, R. A., R. Hull, and S. Falkow. 1983. Molecular cloning and physical characterization of a chromosomal hemolysin from *Escherichia coli*. *Infect. Immun.* **42**:178-186.

Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow. 1981. Hemolysin contributes to virulence of extraintestinal *E.coli* infections. *Nature (London)* **294**:665-667.

Welch, R. A., and S. Pellett. 1988. Transcriptional organization of the *Escherichia coli* hemolysin. *J. Bacteriol.* **170**:1622-1630.

Wells, J. G., B. R. Davis, I. K. Wachsmuth, L. W. Riley, R. S. Remis, R. Sokolow, and G. K. Morris. 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J. Clin. Microbiol.* **18**:512-520.

White-Ziegler, C. A., M. L. Hill, B. A. Braaten, M. W. van der Woude, and D. A. Low. 1998. Themoregulation of *Escherichia coli pap* transcription: H-NS is a temperature-dependent DNA methylation blocking factor. *Mol. Microbiol.* **28**:1121-1137.

Whittam, T. S. 1998. Evolution of *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E.coli* strains, p. 195-209. *In* J. B. Kaper, A. D. O'Brien (ed.), *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E.coli* strains. ASM Press, Washington, D. C.

Whittam, T. S., I. K. Wachsmuth, and R. A. Wilson. 1988. Genetic evidence of clonal descent of *Escherichia coli* 0157:H7 associated with hemorrhagic and hemolytic uremic syndrome. *J. Infect. Dis.* **157**:1124-1133.

Whittam, T. S., M. L. Wolfe, I. K. Wachsmuth, F. Ørskov, I. Ørskov, and R. A. Wilson. 1993. Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. *Infect. Immun.* **61**:1619-1629.

Wieler, L. H., T. K. McDaniel, T. S. Whittam, and J. B. Kaper. 1997. Insertion site of the locus of enterocyte effacement in enteropathogenic and enterohemorrhagic *Escherichia coli* differs in relation to the clonal phylogeny of the strains. *FEMS Microbiol. Lett.* **156**:49-53.

Williams, R. M., Rimsky, and H. Buc. 1996. Probing the structure, function, and interactions of the *Escherichia coli* H-NS and StpA proteins by using dominant negative derivatives. *J. Bacteriol.* **178**:4335-4343.

Willshaw, G. A., S. M. Scotland, H. R. Smith, T. Cheasty, A. Thomas, and B. Rowe. 1994. Hybridization of strains of *Escherichia coli* 0157 with probes derived from the *eaeA* gene of enteropathogenic *E. coli* and the *eaeA* homolog from a vero cytotoxin-producing strain of *E. coli* 0157. *J. Clin. Microbiol.* **32**:897-902.

Winsor, D. K., Jr., S. Ashkenazi, R. Chiovetti, and T. G. Cleary. 1992. Adherence of enterohemorrhagic *Escherichia coli* strains to a human colonic epithelial cell line (T84). Infect. Immun. **60**:1613-1617.

Woolwine-Ciacchi, F., I. C. Blomfield, S. H. Richardson, and S. B. Mizel. 1998. Salmonella flagellin induces tumor necrosis factor alpha in a human promonocytic cell line. Infect. Immun. **66**:1127-1134.

Yam, W. C., D. N. Tsang, T. L., Que, M. Peiris, W. H. Seto, and K. Y. Yuen. 1998. A unique strain of *Escherichia coli* 0157:H7 that produces low verocytotoxin levels not detected by use of a commercial enzyme immunoassay kit. Clin. Infect. Dis. **27**:905-906.

Yamashino, T., M. Kakeda, G. Ueguchi, and T. Mizuno. 1994. An analogue of the *dnaJ* molecular chaperone whose expression is controlled by σ^S during the stationary phase and phosphate starvation in *Escherichia coli*. Mol. Microbiol. **13**:475-483.

Zadik, P. M., P. A. Chapman, and C. A. Siddons. 1993. Use of tellurite for the selection of verocytotoxigenic *Escherichia coli*. J. Med. Microbiol. **39**:155-158.

Zhang, A., S. Rimsky, M. E. Reaban, H. Buc, and M. Belfort. 1996. *Escherichia coli* protein analogs StpA and H-NS: regulatory loops, similar and disparate effects on nucleic acid dynamics. EMBO J **15**:1340-1349.

Zuber, F., D. Kotlars, S. Rimsky, and H., Buc. 1994. Modulated expression promoters containing upstream curved DNA sequences by the *Escherichia coli* nucleoid protein H-NS. Mol. Microbiol. **12**:23-240.

Zhu, C., J. Harel, M. Jacques, C. Desautels, M. S. Donnenberg, M. Beaudry, and J. M. Fairbrother. 1994. Virulence properties and attaching-effacing activity of *Escherichia coli* 045 from swine postweaning diarrhea. Infect. Immun. **62**:4153-4159.

